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Vitamin D and Cardiovascular Disease

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September 2016

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List of commonly used abbreviations

| | |
|------------|--|
| 25(OH)D | 25 hydroxy-calciferol |
| AIx | Augmentation index |
| ARG | Arteriograph |
| BEST-D | Biochemical Efficacy and Safety Trial of vitamin D |
| BMI | Body mass index |
| CAD | Coronary artery disease |
| CRP | C-reactive protein |
| CVD | CVD |
| DBP | Diastolic blood pressure |
| HF | Heart failure |
| HR | Heart rate |
| IHD | Ischaemic heart disease |
| IOM | Institute of Medicine |
| IU | International units |
| LV | Left ventricle |
| Mcg | Micrograms |
| MI | Myocardial infarction |
| nmol/L | Nanomol per litre |
| PWV | Pulse wave velocity |
| SBP | Systolic blood pressure |
| UVB | Ultraviolet B rays |
| VDBP | Vitamin D-binding protein |
| Vitamin D2 | Ergocalciferol |
| Vitamin D3 | Cholecalciferol |

Acknowledgements

The work in this thesis is a result of the collaborative efforts of several teams based at the Clinical Trial Services and Epidemiological Service Unit (CTSU), University of Oxford which included the Information Technology (IT) team, the Wolfson Laboratory team, the statisticians, the administrators and the nurses. I am grateful to the Whitehall study resurvey participants, the participants of the BEST-D trial and the trial team who made this work possible.

My role included a literature review of the prospective studies (Chapter 3) which helped interpret data from the prospective Whitehall study we conducted (Chapter 2). I was involved with the BEST-D trial from the start, with recruitment of participants and study nurses, writing the revised protocol for BEST-D to incorporate measures to evaluate effects of vitamin D on CVD risk markers, planning and design of the trial, and in seeking trial approval from regulatory authorities. I also worked in developing the trial protocols, SOPs, and organised training for the research team. I contributed to the two successful grant applications from the British heart foundation (BHF).

Importantly, I am indebted to Professor Jane Armitage and Professor Robert Clarke, my supervisors at CTSU, Oxford for providing an opportunity to work with them. Their guidance, generosity and understanding during my research fellowship helped develop this project. I am fortunate to have worked and gained much from the depths of their experience. The brain storming sessions were great learning opportunities in skills that varied from the attention to detail and the scientific critique, to the balance between scientific rigour and the pragmatic. I am grateful to Professor Sarah Lamb, who helped with the supervision of this thesis, despite the difficulties posed with distance. The diverse inputs from other researchers at the CTSU helped me understand the application of scientific research for public health benefit.

The successful completion of this research project was largely down to a core team which included Professor Jane Armitage, Professor Robert Clarke, Mrs Jenny Sayer - the BEST-D administrator, Dr Harold Hin - general practitioner at Banbury, and me. The

great working dynamics helped ensure sustained efficiency and momentum and a strategy that adapted with purpose. I am certain this has made this thesis (and the project on vitamin D) both interesting and worthwhile for time to come. I am grateful to my fellow colleagues in the Heart Studies Group for their support. The British Heart Foundation (BHF), the BHF Clinical Research Excellence, Oxford, and the CTSU provided financial support for this work; for this I am also grateful.

Last but not least, the understanding, support and patience from my family were important and immeasurable for the completion of this thesis – my sincere thanks.

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Medicine. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented was carried out by the author except in the cases outlined below:

The analysis of the Whitehall resurvey was led by Professor Robert Clarke in discussion with the other authors of the paper. The statistical analyses for the Whitehall study and the meta-analyses were undertaken by Associate Professor Jon Emberson (Senior Statistician at CTSU, University of Oxford). Dr Emberson with help from Jim Halsey (CTSU, University of Oxford) prepared and undertook the analysis of the BEST-D trial findings. The BEST-D trial visits were conducted by Research nurses, Enid Frost, Lynn Peach and Caroline Boulton, under supervision of the local investigator, Dr Harold Hin (General Practitioner, Banbury).

Parts of this thesis have been published or submitted for publication:

1. Tomson J, Emberson J, Hill M, Gordon A, Armitage J, Shipley M, et al. Vitamin D and risk of death from vascular and non-vascular causes in the Whitehall study and meta-analyses of 12 000 deaths. *Eur Heart J*. 2013; 34(18): 1365-74.
2. Clarke R, Newman C, Tomson J, Hin H, Kurien R, Cox J, Lay M, Sayer J, Hill M, Emberson J, Armitage J for the BEST-D Collaborative Group. Estimation of the optimum dose of vitamin D for disease prevention in older people: rationale, design and baseline characteristics of the BEST-D trial. *Maturitas*. 2015 Apr;80(4):426-31.

3. Hin H, Tomson J, Newman C, Kurien R, Lay M, Cox J, Sayer J, Hill M, Emberson J, Armitage J, Clarke R for the BEST-D Collaborative Group. Optimum dose of vitamin D for disease prevention in older people: BEST-D trial of vitamin D in primary care (Submitted).

Summary

Background: Cardiovascular disease (CVD) is the leading cause of death worldwide. Vitamin D is important for bones and for other body functions. Whether insufficient vitamin D causes CVD is unclear. Previous trials of vitamin D were unable to evaluate effects on CVD.

Aims This thesis will (i) review the literature on vitamin D and CVD; (ii) evaluate the observational associations between plasma 25(OH)D levels and CVD; and (iii) describe the design and results of the BEST-D trial.

Methods (i) Associations between baseline 25(OH)D levels and cause-specific mortality were evaluated in the Whitehall Resurvey of survivors undertaken in 1995, and findings included in a meta-analysis of similar studies. (ii) The BEST-D study was a randomised trial in older healthy volunteers of the effects of two doses of vitamin D₃ (4000 IU or 2000 IU daily) compared to placebo, on blood 25(OH)D concentrations and CVD risk factors including blood pressure and arterial stiffness.

Results (i) The Whitehall Resurvey of 5409 men with mean age of 77 years, among whom there were 3215 deaths showed an approximately linear (log-log scale) inverse association of plasma 25(OH)D concentrations and both CVD and non-vascular causes of death between 30 to 90 nmol/L. The meta-analysis confirmed the associations of 25(OH)D with CVD mortality. (ii) The BEST-D trial showed marked increases in 25(OH)D blood concentrations but no effects of taking higher doses of vitamin D₃ for 12 months on blood pressure or arterial stiffness, compared to placebo.

Conclusions

Plasma 25(OH)D is inversely associated with both CVD and non-vascular mortality. No effects were found after oral intake of vitamin D₃ on blood pressure or arterial stiffness after 1 year. Randomised trials using adequate doses of vitamin D₃ are needed, to evaluate causal effects of taking vitamin D on CVD outcomes.

Total Word count: 29,663 (excluding references, Appendix, Tables and Figures)

Abstract word count: 292

Number of figures: 21

Number of tables: 22 Colour pages:5

Chapter 1 Introduction: Why examine Vitamin D and cardiovascular disease

Rationale and Overview of thesis

Low vitamin D levels are prevalent world-wide and there is a re-emergence of diseases like rickets and osteomalacia, which are associated with vitamin D deficiency. CVD is the single most common cause of death worldwide, with a number of established risk factors identified.¹ However, identifying newer CVD risk factors remains relevant and may help with the understanding of CVD pathophysiology and could lead to newer therapeutic targets for prevention or treatment of CVD. Vitamin D insufficiency may be a potential new risk factor for CVD, and is potentially modifiable.

Vitamin D is important for maintaining blood calcium levels and for healthy bones,² but whether low levels of vitamin D, assessed by measuring plasma 25-hydroxyvitamin D [25(OH)D –the main storage form of vitamin D],^{2, 3} are causally related to CVD is unclear. New research shows that vitamin D influences about 10% of the human genome⁴ and those vitamin D receptors have been described in almost all tissues of the human body.^{5, 6} In tissues other than the liver and the kidneys, there is evidence of vitamin D metabolism which suggests a wider role for vitamin D beyond calcium regulation and bone health.⁷

Observational studies of vitamin D (discussed in the next chapter) demonstrate higher rates of CVD deaths with increasing latitude from the equator, at higher altitudes and during winter.⁸ These observed variations in CVD death rates are not fully explained by

our current understanding of the pathophysiology of CVD or by its established risk factors. One possible explanation may be that these differences are related to the variation in circulating vitamin D levels according to the availability of sunlight. Sun exposure of skin is the main source of vitamin D for the human body and accounts for its seasonal variation. People living in regions at the extremes of latitude from the equator have reduced ultra-violet B (UVB) rays availability. The UVB availability is lowest during winter in these regions as the sun's radiation has to travel through a greater thickness of the atmosphere given the low angle of the sun in winter (in relation to its position in summer); this leads to reduced or no vitamin D production and could be linked to the higher CVD deaths. If a causal link between low vitamin D and CVD is established, modifying vitamin D levels could be an effective public health measure for prevention of CVD. Increasing vitamin D levels through vitamin D supplements or fortification is feasible and cost-effective.

Prospective studies report inverse associations of 25(OH)D levels with CVD risk factors and CVD deaths (reviewed in the next chapter), but the shape and strength of these associations, the consistency of the observations and the generalizability of the findings to the broader general population remain unclear. However, the evidence from clinical trials and genetic studies, reviewed in the next Chapter, is inconclusive and there is a case for more studies to test the causality of the reported associations.

This chapter also introduces and discusses the concept of 'optimal' 25(OH)D concentrations, especially with a focus on cardiovascular and other health outcomes, and the controversies surrounding its definition.

The relationship between circulating 25(OH)D concentrations and cause-specific mortality was explored in a prospective analysis of the Whitehall resurvey participants (Chapter 3).⁹ Inverse log-linear associations of baseline 25(OH)D concentrations with both CVD and non-CVD causes of death were observed in this study. There was a lack of clarity if these were consistent and comparable to the findings from the other prospective studies reporting on CVD and all-cause mortality. Hence the Whitehall resurvey findings were included in meta-analyses of prospective studies (Chapter 4) that predominantly evaluated associations of baseline 25(OH)D concentrations with either all-cause mortality and CVD mortality, and found that these reported associations from the Whitehall study were consistent and congruent, and comparable to studies that included participants living in other regions of the world.⁹

Evidence from randomised-controlled trials of vitamin D on the effects of supplementation on cardiovascular disease outcomes is limited. Most previous trials of vitamin D supplementation were designed to evaluate effects of on fractures and falls, or muscle strength and balance, and were conducted predominantly among the older people. These trials also typically assessed doses equivalent to vitamin D 400 IU to 800 IU daily which would have been insufficient to raise 25(OH)D levels to concentrations associated with the lowest risks of CVD and all-cause mortality observed in the prospective studies, and hence may have lacked statistical power to demonstrate effects on CVD outcomes. As alluded to previously, although the optimal levels of 25(OH)D are a matter of debate, the early evidence from various observations suggest that plasma levels of 80-90 nmol/L may be 'optimal' for multiple health outcomes including CVD.⁹⁻¹¹ Hence, the previous trials of vitamin D were unlikely to have achieved

significant differences in plasma concentrations of 25(OH)D between the treatment arms when using the typically small equivalent daily doses, and hence would not have been able to detect differences on CVD outcomes. Further well-designed, large trials using sufficient doses of vitamin D supplements¹² are needed to evaluate the role of vitamin D supplements on CVD outcomes.

Before designing another trial of vitamin D supplements, the dose of vitamin D supplement that would achieve concentrations considered to be the 'optimal' plasma 25(OH)D levels, needs to be established. Typically a dose of 400 units of vitamin D will raise 25(OH)D levels by only 7-10 nmol/L¹³ and so to raise blood concentrations 25(OH)D levels, associated with the lowest risks for multiple health outcomes including CVD (i.e. >80-90 nmol/L),⁹ doses of 2000 IU daily or more may be required^{11, 14, 15} Data from the National survey suggests that older adults have plasma 25(OH)D of about 50-55 nmol/L at the end of summer and the levels fall further in winter.¹⁶ The safety, efficacy and tolerability of such higher daily doses of vitamin D3 supplements (>2000 IU daily) especially when taken for longer periods have not been adequately assessed.¹³ Chapter 5 of this thesis describes the methods and design of a dose-finding trial of vitamin D called BEST-D (Biochemical efficacy and safety of vitamin D) evaluating effects of two doses of daily vitamin D3 (cholecalciferol) 2000 IU and 4000 IU, versus placebo on plasma 25(OH)D levels.¹⁷ The results of BEST-D are reported in Chapter 6.

To reliably establish a causal relationship between low vitamin D and CVD, appropriately designed randomised trials using adequate doses of vitamin D supplements are necessary, and some are already underway. However, the earliest results from these

trials evaluating effects on CVD outcomes are not expected until at least 2017.¹² Given this context, assessing the effects of vitamin D on CVD risk markers may provide some insight into potential effects of vitamin D supplements on CVD outcomes, if any. Some observational studies have suggested that low vitamin D is associated with a higher blood pressure and higher arterial stiffness but the effect of supplementing with vitamin D is uncertain. We tested the effects of taking two daily doses of vitamin D3 (2000 IU and 4000 IU daily) versus placebo for 1 year on blood pressure and arterial stiffness among the participants in the BEST-D trial. Thus this thesis will seek to explore whether low vitamin D could cause CVD and test the effects of the vitamin D on CVD risk markers. The analysis of the findings, discussed in Chapter 7 will help inform the design of a future randomised trial of vitamin D evaluating the effects of vitamin D on CVD outcomes.

Summary

Evidence from observational studies of vitamin D, and the emerging laboratory and genetic evidence on the biology of the vitamin provides a strong basis for exploring a possible role for low vitamin D as a risk factor in the development of CVD. If low vitamin D causes CVD, modifying circulating levels of vitamin through supplementation could become an effective public health measure for preventing CVD.

Aims of thesis

The main aims of this thesis are to:

1. Review observational evidence from prospective studies reporting associations of blood 25(OH)D and CVD;
2. Review the results from randomised trials of vitamin D and CVD outcomes;
3. To describe the design of a dose-finding trial of vitamin D called BEST-D, and report the effects of taking vitamin D supplements for 12 months on blood pressure (CVD risk factor) and arterial stiffness (surrogate marker for CVD).

Outline of Chapters

Chapter 1: Introduction: Why examine vitamin D and CVD

Chapter 2: Vitamin D and CVD

This chapter reviews the published literature on observational studies and clinical trials of vitamin D and cardiovascular outcomes.

Chapter 3: Vitamin D and cause-specific mortality in the Whitehall resurvey

This chapter presents the findings of a prospective study reporting on associations between baseline plasma 25(OH)D and cause-specific mortality over a 13-year follow-up, among participants in the Whitehall resurvey.

Chapter 4: Meta-analyses of prospective studies of vitamin D and the risks of CVD and all-cause mortality

The findings from the Whitehall resurvey (Chapter 3) are put in perspective by including these in meta-analyses of other prospective studies reporting on baseline 25(OH)D and CVD or all-cause mortality.

Chapter 5: The Biochemical Efficacy and Safety Trial of vitamin D (BEST-D) – design and methods to evaluate the effects of vitamin D on blood pressure and arterial stiffness.

The design of this dose-finding trial of vitamin D and the methods for evaluating the effects of vitamin D supplements on blood pressure and arterial stiffness among participants of the Biochemical Efficacy and Safety Trial of vitamin D (BEST-D), is discussed.

Chapter 6: Effects of vitamin D on blood pressure and arterial stiffness

The effects of taking high dose vitamin D₃ supplements daily for a year, on blood pressure and CVD are presented.

Chapter 7: Analyses and Conclusion

Role of the author in this thesis

All the work presented in this thesis is my own conducted under the supervision of Professor Jane Armitage and Professor Robert Clarke and with remote supervision from Professor Sally Lamb and as part of a larger body of collaborative projects on vitamin D, done at the CTSU, University of Oxford.

I joined the CTSU as a Clinical Research Fellow to help with the running of the on-going large-scale CVD trials, and was given the opportunity to get involved in the planned BEST-D study. The study was designed as a pilot dose-finding study in preparation for a large randomised study to assess the effect of vitamin D supplementation on risk of fractures. With my background in cardiovascular medicine, and interest in the effect of vitamin D on CVD, in discussion with my supervisors, we decided to evaluate the effects of vitamin D supplements on cardiovascular risk factors in the planned study and modified the protocol. I was interested on the effects of weather and CVD and hence keen to explore the effects of vitamin D on CVD.

Before the trial was established, I conducted a literature review of the prospective studies of vitamin D reporting on the associations of blood 25(OH)D concentrations and risk of deaths from CVD and all-cause mortality, and also worked with colleagues on evaluating the relationship between baseline plasma 25(OH)D levels and cause-specific mortality in a subset of survivors of The Whitehall study who were resurveyed in 1997 by Professor Clarke. The data from Whitehall was interpreted in the context of a meta-analysis of similar prospective studies of vitamin D that I undertook. Dr Jon Emberson (Senior statistician, CTSU), undertook the statistical analyses for this work), and

laboratory support was provided by Dr Michael Hill (Laboratory Scientific Director, CTSU) and his team at Wolfson laboratory, CTSU. I worked with Dr Harold Hin, (General Practitioner, Hightown Surgery, Banbury) who was the local investigator for the BEST-D trial and who helped with trial management at Banbury, including help with identifying and recruiting suitable study nurses and participants.

The protocol for BEST-D was revised in the light of these findings to incorporate measures of the effects of vitamin D on CVD risk markers. I was involved in the planning, and design of the trial, and in seeking the approval from the relevant Ethics Committees and the Medicines and Healthcare products Regulatory Agency (MHRA). I evaluated the assessment methods used in the trial, and its logistics with help from Jenny Sayer (trial administrator). I worked with senior IT colleagues at CTSU (Dr Mike Lay – head of Heart Studies Group programming team; Jolyon Cox – senior researcher; and Rijo Kurien - programmer), in developing the be-spoke trial software. This was based on the questionnaire outlines I designed and developed (with input from Jane Armitage and Robert Clarke) for the trial. I also developed the trial-specific training materials and trial ‘standard operating procedures’ (SOP).

We secured two grants to support the work on CVD from the British heart foundation (BHF). The research proposal entitled ‘Does vitamin D supplementation improve markers of inflammation, innate immunity and arterial stiffness?’ developed in collaboration with the Wellcome Trust Centre for Human Genetics, Oxford, secured a BHF Project grant (PG/12/32/29544) for a sum of £ 112,140. This grant supports the work on evaluating effects of vitamin D supplements on genetic markers of

inflammation and immunity (not part of this thesis), and the effects on arterial stiffness. The BHF Centre for Research Excellence (BHF CRE), Oxford provided a grant for £ 50,000 to evaluate 'Effects of vitamin D supplements on cardiac contractile function' in the BEST-D trial. This grant supports the evaluation of cardiac function of participants in the BEST-D trial at the end of the study using echocardiography (not included in this thesis). The research included in this thesis is from work where I have had a major contribution.

Publications relating to this thesis

1. Tomson J, Emberson J, Hill M, Gordon A, Armitage J, Shipley M, et al. Vitamin D and risk of death from vascular and non-vascular causes in the Whitehall study and meta-analyses of 12 000 deaths. *Eur Heart J*. 2013; 34(18): 1365-74 (Appendix 1).
2. Clarke R, Newman C, Tomson J, Hin H, Kurien R, Cox J, Lay M, Sayer J, Hill M, Emberson J, Armitage J for the BEST-D Collaborative Group. Estimation of the optimum dose of vitamin D for disease prevention in older people: rationale, design and baseline characteristics of the BEST-D trial. *Maturitas*. 2015 Apr;80(4):426-31.
3. Hin H, Tomson J, Newman C, Kurien R, Lay M, Cox J, Sayer J, Hill M, Emberson J, Armitage J, Clarke R for the BEST-D Collaborative Group. Optimum dose of vitamin D for disease prevention in older people: BEST-D trial of vitamin D in primary care (In submission).

Chapter 2 : Vitamin D and Cardiovascular disease

The burden of cardiovascular disease

Cardiovascular disease is the leading cause of death globally,¹ and accounts for more than a third of all deaths. Age-adjusted CVD death rates in developed countries such as the UK and USA are falling¹⁸⁻²⁰, thought to be largely due to progress in implementing CVD preventive measures such as smoking cessation and control of established CVD risk factors. However, CVD death rates in the developing countries and emerging economies are rising¹ and CVD prevention remains a key challenge for public health globally. This chapter reviews the literature on the relationship between CVD and vitamin D.

Vitamin D as a risk factor for CVD

The pathophysiology of CVD is not fully understood and is not entirely explained by classical CVD risk factors.²¹⁻²³ [In this thesis, CVD refers to coronary heart disease (CHD) and other cardiovascular disease e.g. stroke and peripheral arterial disease]. For instance, existing risk factors for CHD are thought to explain between 50-75% of all incident coronary heart disease (CHD).²¹⁻²³ Hence, there is a need to evaluate other potential risk factors for CHD and/or CVD. It is also known that CVD can occur in people with either some or no established CVD risk factors,^{18 24} i.e. the risks of CVD is not zero in those with 'low risk scores for CVD'. Although this may be explained by the effects of residual confounding, where pre-existing cardiovascular risk factors are not fully accounted for due to imprecise measurements or within-person variation, the unexplained risk may be due to the existence of other hitherto unidentified risk factors for CHD. Such novel risk factors if identified, may help explain the inconsistencies in the

literature of observed variation in CVD rates, such as the low incidence of CVD among people living in France compared to elsewhere in Europe despite similar prevalence of CVD risk factor levels;²⁵ or the 50% higher risk of cardiovascular deaths among South Asians living in the UK compared to Caucasians.^{26, 27} Finally, new risk factors would enable us to better understand CVD pathophysiology and present newer targets for prevention and therapy.

Evidence from recent research suggest vitamin D has effects on many tissues in the body as vitamin D receptors have been detected in almost all tissues.⁵ Vitamin D is also now shown to be metabolised in tissues other than those classically described, such as the liver, kidneys and bone, indicating a role in the wider human physiology.^{7, 28, 29} Genetic studies confirm vitamin D influences a tenth of the human genes.⁴ Hence, it is possible that maintaining plasma levels higher than 25 nmol/L may be important for human health in general and for CVD.

Additional evidence from ecological studies supports further evaluation of the role of vitamin D in the development of CVD. Ecological studies have reported inverse associations between deaths from ischaemic heart disease (IHD) and increasing latitude from the equator (**Figure 2-1**),^{8, 30, 31} but this is weak evidence for it being an effect of vitamin D. This trend is consistent with the relative lack of UVB availability from sunlight, as there is reduced UVB available with increasing distance from the equator. Several other studies also report higher rates of CVD deaths in winter compared to summer.^{32, 33} The reduction in UVB is worse during winter and more pronounced at the extreme of latitudes from the equator, as the UVB rays have to travel longer through an

increased thickness of the atmosphere, given the shallower angle at which the sun rays would fall at this time of the year.³⁴ Hence, differences in circulating vitamin D levels may account for some or all of the observed differences in CVD death rates.

Vitamin D metabolism

The metabolism and physiology of vitamin D has been comprehensively reviewed elsewhere,^{2, 35, 36} but an outline of the relevant aspects is described below (**Figure 2-2**).

Vitamin D is an essential vitamin and can be synthesised in the human body after exposure of the skin to ultraviolet B rays (UVB) from sunlight (UVB rays that have a wavelength spectra between 290–315 nm).^{2, 37} The UVB rays convert 7-dehydrocholesterol, a precursor of cholesterol present in the skin, to pre-vitamin D₃ and subsequently to vitamin D₃ (cholecalciferol). The process is both light and temperature-sensitive. Prolonged sunlight exposure or temperature converts vitamin D₃ to other metabolites. Hence, production of vitamin D is regulated by both temperature and light changes.

Vitamin D₂, or ergocalciferol, is the main form of vitamin D obtained from plant sources and has less biological activity than vitamin D₃. Vitamin D₃ is also found in some foods like fatty fish and egg yolks. But diet is a poor source of the vitamin in general, as only small quantities of vitamin-rich foods are consumed. While some countries fortify foods with vitamin D, fortification is not currently implemented in the UK.^{38, 39} Both vitamin D₂ and D₃ are transported in blood predominantly bound to the vitamin D-binding protein (VDBP), and metabolized to 25-hydroxyvitamin D [25(OH)D] by cytochrome

P450 dependant enzymes mainly, but not exclusively, in the liver. One of the liver enzymes, CYP27A1 is the most widely described catalyst promoting this hydroxylation step. Excess 25(OH)D is bound to vitamin D-binding protein in blood, the affinity of this binding capacity is variable and could impact on the bioavailability; some 25(OH)D may also be stored in fat and muscle, and released when required. Plasma 25(OH)D has a half-life of 2-3 weeks. Blood levels of 25(OH)D are widely used as a measure of vitamin D status in humans, rather than the active form of the vitamin D, which is 1,25(OH)D.⁴⁰

The active form of vitamin D, or calcitriol [1,25(OH)D], is formed after a further hydroxylation of 25(OH)D, predominantly in the kidneys. This step is catalysed by CYP27B1, an enzyme once thought to be present only in the kidneys, but which now has been shown to be present in several cell types, suggesting that vitamin D metabolism occurs in other tissues and could have actions there. For instance, CYP27B1 is present in macrophages and white cells and could suggest a role for vitamin D in immunity;^{28, 41} Vitamin D has also important roles in the cell cycle influencing cell division, maturation, differentiation and death.⁴² Due to these varied actions in multiple tissues and the local metabolism and regulation, some consider vitamin D as a pro-hormone.

The active vitamin, 1,25(OH)D, binds mainly to a nuclear receptor (vitamin D receptor, VDR),⁶ which in turn bind to the vitamin D response elements (VDRE) in genes. VDR belongs to the steroid/thyroid superfamily of receptors, and after binding with 1,25(OH)D, brings about transcriptional changes through DNA and micro-RNA mediated transcriptional responses. The VDR receptors are of different shapes and are important in mediating different responses to 1,25(OH)D concentrations,⁴³ such as the rapid

response, which typically causes increased gut absorption of calcium; or the slower response which includes the actions of genetic and transcriptional modification and takes longer to manifest.^{4-7, 44} The VDR is found in cardiac myocytes as well as immune cells and platelets. Further, genetic expression is influenced and modified by other co-activators and suppressors and possibly micro-RNAs.⁴⁵ VDR can bind to both 1,25(OH)D and 25(OH)D. Hence, 25(OH)D can initiate some physiological actions of vitamin D. Both 25(OH)D and 1,25(OH)D can trigger the 'rapid response', albeit the physiological role of this response is still under investigation.

Production of active vitamin D is also regulated by the parathyroid hormone (PTH), which stimulates the renal production of 1,25(OH)D when circulating 25(OH)D is low (**Figure 2-2**). Vitamin D and parathyroid hormone (PTH), an 84-aminoacid polypeptide secreted by the parathyroid glands located in the neck, form parts of a feedback mechanism that controls bone and calcium metabolism. The blood levels of the active vitamin D, 1,25(OH)D, are normally about a thousandth of the concentration of circulating 25(OH)D. The levels of 25(OH)D determine plasma levels of active vitamin D [i.e. the higher circulating 25(OH)D, the higher the levels of 1,25(OH)D]. However, 1,25(OH)D can down-regulate its own production locally.³⁶

The main physiological action of vitamin D is to maintain blood calcium levels within the normal range by promoting intestinal absorption and the renal re-absorption of calcium. Plasma concentrations of calcium are tightly controlled in the body to ensure a steady uninterrupted supply (an important role for vitamin D in vertebrates). When plasma

calcium levels fall, a number of mechanisms ensure adequate circulating levels of the ion (discussed below).

A number of other factors influence circulating vitamin D levels. From an evolutionary perspective, melanin may be the most important.⁴⁶ Melanin is a pigment molecule present in the superficial layers of the skin and gives it the dark colour, and depending on the distribution reduces the amount of UVB rays reaching the deeper layers of skin where vitamin D synthesis takes place. Hence, for a similar amount of UV light exposure lower vitamin D levels are seen in people with more skin pigmentation. There is evidence that fairer skin colour is an adaptation to enable endogenous production of vitamin D with migration of early man from equatorial regions to higher and lower latitudes.⁴⁶ Further, the genes activated by vitamin D happen to be located in areas of the genome, protected and passed on over time with evolution – genes of positive selection.⁴ Hence, it is unclear if human beings would adapt to lower plasma 25(OH)D concentrations when migrating to areas of less UVB availability, or whether this adaptation has already occurred if any over the years.

Calcium metabolism

Calcium is vital for many functions in the human body, especially for bones, muscle contraction, nerve and cardiac impulse conduction and intra-cellular signalling.^{47, 48} The vast majority of calcium is present in bone as a calcium-phosphate complex, while the remainder constitutes only 1% of calcium in the human body and is predominantly in a circulating form.

Circulating calcium can be in the ionised or non-ionised forms.^{48, 49} The non-ionized calcium is bound to calcium-binding proteins like albumin and globulin in serum and calmodulin within the cell. The major ionic calcium complexes in serum are calcium phosphate, calcium carbonate, and calcium oxalate. However, it is the ionised calcium that is important in the regulation of serum calcium levels (and forms 50% of all circulating calcium), and this in turn is closely regulated through a negative feedback mechanism as described above.

Parathyroid hormone is an 84-amino acid polypeptide secreted by the parathyroid glands in response to a fall in circulating calcium concentration and through a negative feedback mechanism influenced by circulating concentrations of phosphate and fibroblast growth factor-23 (FGF23).^{35,50} It acts by increasing renal reabsorption of calcium and stimulating the 1,25(OH)D production which in turn increases calcium absorption from the intestine and promotes bone remodelling. Additionally, PTH regulates skeletal growth factors which help with building bone tissue.

A fall in serum calcium ions inactivates receptors situated in the parathyroid cells causing an increase in PTH secretion from the gland.⁵¹ This in turn helps restore serum calcium through bone resorption secondary to the increased osteoclast activity, by increasing the renal tubular calcium reabsorption and reduction in excretion, and an increase in gut absorption. The increased PTH levels also promote hydroxylation of 25(OH)D and the consequent increase in 1,25(OH)D, the active form of vitamin D.

The amount of calcium excreted in a day is typically around 200 mg/d for adults. The net absorption of calcium from the gut reaches 200 mg at a calcium supplement intake of around 1200 mg/d, which is the current recommendation for calcium intake in adults.

The proportion of calcium absorbed from the gut is positively correlated with circulating 25(OH)D concentrations. Supplementation with vitamin D can significantly increase the actively absorbed fraction of calcium from the gut, even without achieving higher 1,25(OH)D levels.^{49, 52} To explain this, a self-regulatory model of calcium metabolism has been proposed, where sufficient calcium and 25(OH)D levels determine the rate and efficiency of calcium absorption.^{49, 52} Thus, when 25(OH)D levels are suboptimal, intestinal calcium absorption in response to adequate calcitriol levels is reduced perhaps through the selective influences on the active calcium transporters in the gut wall. When vitamin D is sufficient (i.e. in the presence of optimal circulating 25(OH)D levels) there is a down-regulation of 1,25(OH)D induced calcium absorption.

Optimal levels of vitamin D

Vitamin D adequacy is assessed by measuring blood levels of 25(OH)D and deficiency is usually defined as blood levels <25(OH)D nmol/L and below such levels there is an increasing risk of developing rickets in children and osteomalacia in adults² (although the Endocrine Societies have proposed using a higher cut-off of <50 nmol/L).⁵³ In this thesis, I define insufficient levels as a plasma concentration of circulating 25(OH)D between 25-75 nmol/L (rationale explained below; for values in ng/ml, divide values by 2.5).

The significance of higher plasma concentrations than 25 nmol/L for health is unclear, but it is currently proposed that a plasma concentration of 25(OH)D of at least 50 nmol/L is needed for optimal bone health.⁵⁴ Maintaining higher plasma levels of

25(OH)D above the deficiency range may also be relevant to other health outcomes, such as CVD, cancer and infections.⁵⁵ However, there is still no consensus on the definition of 'optimal circulating levels of 25(OH)D' as there is currently a debate on what constitutes optimal levels.^{53, 55}

Biochemical methods for defining optimal 25(OH)D concentrations have been proposed but are not perfect. One widely used method is to use the level of 25(OH)D concentration above which no further suppression of PTH concentrations occur through the negative feedback loop mechanism (typically at around 75 nmol/L concentrations), and to use this as an indicator of optimal levels.^{2, 51} However, in children and during puberty, PTH levels are found to be higher compared to those in adults. Renal dysfunction, exercise and the time of the day when samples are taken, all cause variations in estimated PTH levels and hence this method is imperfect. The blood 25(OH)D levels at which no further increases in serum calcium is observed was also proposed but rejected.⁵⁶ Serum calcium is tightly regulated and is controlled through the influences of multiple hormonal systems as described earlier, and hence the response tends to be variable. For instance, high PTH levels (such as in hyperparathyroidism) could raise calcium concentrations independent of calcitriol concentrations.⁵⁶ One other proposed method is to use the level of 25(OH)D concentration beyond which, a further rise fails to elicit an increase in 1,25(OH)D concentrations, as being the sufficient level. However, concentrations of 1,25(OH)D fall only when 25(OH)D levels are very low; hence the relationship between 25(OH)D concentrations and 1,25(OH)D levels is not linear. Further, the concentration of 1,25(OH)D varies due to factors which include calcium intake, presence of certain

granulomatous diseases or pregnancy (both of which raise 1,25(OH)D concentrations).⁵⁷ Further, changes in 1,25(OH)D concentrations do not seem to correlate well with the biological effects, but appear to correlate better and more consistently with circulating 25(OH)D concentrations. This may mean that some of the vitamin D-mediated actions may also be due to the influences from the circulating 25(OH)D molecules; hence this method is not ideal. Heaney et al,⁵¹ suggested using the 25(OH)D level that results in the maximal intestinal calcium absorption instead, but studies have again not been able to provide consistent verification of a linear relationship.⁵⁸

Current efforts have increasingly focussed on defining optimal levels by establishing the circulating vitamin 25(OH)D concentrations associated with the lowest risks for various health outcomes.^{9, 11, 53} There has been much debate and conflicting evidence on the optimal levels.^{54, 59} The Institute of Medicine (IOM) conducted a systematic review of all vitamin D studies published and concluded that for optimum bone health, 600 IU of vitamin D daily was sufficient for most adults to achieve a plasma level of about 50 nmol/L (20ng/ml), and that these levels were to be considered optimal.⁵⁴ For other health outcomes, the review found limited or inadequate evidence to make recommendations, although effects of vitamin D supplements on other health outcomes were not within the remit or focus of this review.

The prospective observational studies (discussed in Chapters 2 and 4) suggest that blood levels of 75-90 nmol/L are associated with the lowest risks of mortality. For the purposes of this thesis, deficiency is defined as plasma levels of <25 nmol/L; insufficiency as levels greater than 25 nmol/L but less than 75 nmol/L, and optimal

plasma levels as >75 nmol/L but while recognising that these definitions are not fully evidence-based.

Prevalence of vitamin D insufficiency

Insufficient 25(OH)D levels in the general population have been reported worldwide, but there are inconsistencies in the reports especially in the proportion of the population attributed to have insufficiency. Broadly, insufficiency of vitamin D appears to affect between 20-50% of many populations and is more common among people living in countries at the extremes of the hemispheres.⁶⁰⁻⁶⁴ In the United Kingdom, data from the National Diet and Nutrition Survey of British Adults show mean (SD) concentrations of 25(OH)D among older people (> 65 years of age) to be 55.5 (26.9) nmol/L in summer with about half these levels observed during winter.¹⁶ Among the very elderly people living in care homes, the mean levels are 32.8 (15.7) nmol/L. Hence using these definitions, about 5-20% of the population in the UK are deficient of vitamin D and 20-70% could have insufficient levels, and is more common among young adults, older people and ethnic minorities also.

Insufficient levels of 25(OH)D are also not uncommon among people living in the sunnier regions of the world even in countries closer to the equator.⁶⁰ In places like the middle east and some nations in Africa, the reported insufficiency of vitamin D may be related to the social customs, religious practices, costumes worn and specific dietary patterns.⁶³ As low 25(OH)D levels have been reported from every region in the world where estimates were made,^{62, 63} insufficient vitamin D is a concern for public health globally.

Determinants of low concentrations of blood 25(OH)D

Vitamin D deficiency was identified as a problem at the start of the 20th century,^{65, 66} after observations that rickets was common among children living in cities, compared to those inhabiting rural areas. Vitamin D was identified and isolated by Adolph Windaus, and the eventual widespread fortification of food helped eradicate these bone diseases. However, fortification was stopped or reduced in many countries after concerns emerged over hypercalcaemia among young children in England.

There is no single factor that explains the widespread prevalence of low vitamin D levels, and the causes are likely to be multifactorial.^{39, 67, 68 69 70} Factors that reduce measured 25(OH)D concentrations include advancing age, female gender, increased body fat, darker skin type,⁷¹⁻⁷³ low fish oil diet, increased clothing, reduced duration of skin exposure to sunlight, and winter.⁶⁰⁻⁶² Geographic location and lifestyle are important factors contributing to the prevalence of vitamin D insufficiency, but are difficult to modify. Lifestyle factors include the impact of increasing industrialisation and urbanisation with air pollution affecting sun exposure, sun-avoidance behaviour and use of sunscreen to reduce skin cancer risk, indoor work and recreation and religious practices imposing body cover.

In the United Kingdom and for people living in countries farther from the equator (above and below 37 degrees latitude), UVB availability is low during winter (because the UVB rays travel longer through the atmosphere in winter due to the angle of the sun, and as a result the UVB rays get attenuated). Hence the low vitamin D levels seen in the population is worst during winter^{37, 68, 74-76} as no vitamin D is produced despite

sun exposure between the months of October and March in such locations.^{37, 77}

Furthermore, as ultra-violet A (UVA) rays from sunlight are less attenuated than UVB, these may reduce vitamin D₃ already synthesised in the skin.⁶⁸

Smokers have been shown to have lower 25(OH)D circulating levels, but the mechanisms behind this association or the impact of it is unclear.^{78, 79} Other less recognised factors affecting 25(OH)D concentrations include vitamin D binding protein levels and the affinity of its binding, where the differential binding capacity for 25(OH)D is variable and could cause variations in available 25(OH)D. More studies are being conducted to understand the role of this carrier protein.⁸⁰ Increased body fat content, washing,^{81, 82} and the age-associated reduced skin production of vitamin D₃⁸³ are all associated with lower circulating 25(OH)D levels.

Women have lower 25(OH)D levels than men and this is thought to be associated with the higher body fat content.^{84, 85} In a cross-sectional analysis of the Women's health initiative (WHI) trial participants, abdominal obesity was significantly and inversely associated with 25(OH)D levels.⁸⁶ It is not clear however, whether fat acts simply as a store of 25(OH)D¹⁴ and releases 25(OH)D into circulation when needed (similar to the actions of vitamin-D-binding globulins and skeletal muscle),⁴⁰ or whether there are other actions of vitamin D in fat.⁸⁷

Effects of supplementation with Vitamin D on 25(OH) blood levels and safety

Based on the available evidence, the average increase in 25(OH)D concentrations per 400 IU (10 µg) of vitamin D₃ taken orally is about 7-10 nmol/L.^{11, 88} Previous trials

suggest 600-800IU daily is probably sufficient to maintain plasma 25(OH)D levels to >50 nmol/L.⁵⁴ In the UK, mean 25(OH)D levels among people over 65 years of age range between 32 (winter) and 55 (summer) nmol/L;¹⁶ hence daily vitamin D supplements much greater than 600 to 800 IU daily may be needed to achieve and maintain 'optimal' 25(OH)D¹¹ concentrations particularly in certain populations such as older and/or non-Caucasian people. The current daily recommended intake for vitamin D in the UK is 200 IU/day, but since 2012, the Department of Health has recommended that pregnant women, young children between the ages of 6 months to 5 years and older people above the age of 65, should take vitamin D supplements (up to 400 IU daily).⁸⁹

However, wide variations were found in the achieved 25(OH)D concentrations among African-American trial participants taking between 3800 IU and 5000 IU of vitamin D daily,⁹⁰ when compared to other trials which included Caucasian older women,⁹¹ which could have implications when planning dosing of vitamin D supplements for trials, as higher doses of vitamin D may be necessary in certain sub-groups of people such as the obese, the dark-skinned and the elderly in particular.

Daily dosing of vitamin D appears to be more efficacious in raising circulating 25(OH)D plasma concentrations compared to intermittent high doses orally or by injection.⁹² In a meta-analysis by Autier et al,⁹³ wide variations in the achieved plasma concentrations of 25(OH)D were found between studies using similar doses of vitamin D supplements.⁹³ The concomitant use of calcium, especially in those with higher baseline 25(OH)D concentrations resulted in smaller increases in circulating plasma 25(OH)D levels compared to those who did not take concomitant calcium supplements. Further,

vitamin D₃ supplementation was found to be more efficacious than vitamin D₂ in achieving target plasma 25(OH)D response.

Doses of vitamin D up to 4000 IU daily are generally considered safe⁵⁴ and up to 100,000 IU has been given as intermittent doses.⁹⁴ However, there is only limited safety data on the use of daily doses of vitamin D 2000-4000 IU daily and previous dosing trials have been small and of a short duration and used differing modes of administration.^{95, 96}

Some safety data are provided by the long-term fracture prevention trials.⁹⁷⁻⁹⁹ In one large randomised trial bolus doses of 500,000 IU oral vitamin D₃ were administered yearly to 2256 post-menopausal women over 70 years of age, and there was a significant excess in of falls and fractures (RR for fractures, 1.2; 95% CI, 1.00:1.59; P = .047) in the treatment group compared to placebo;¹⁰⁰ but no such hazards were reported with daily dosing of vitamin D supplements.^{101, 102} In the much larger WHI, a higher incidence of renal stones was reported among women taking 400 IU daily compared to placebo (hazard ratio: 1.17; 95% CI: 1.02, 1.34);¹⁰³ but the use of concomitant calcium supplements means that it is not possible to know if it was the effect of vitamin D or the combination; but there were no other significant hazards identified.^{96, 104} Other shorter term trials have not reported major safety concerns when using daily doses of vitamin D supplements of up to 4000 IU daily.

Vitamin D and cardiovascular disease

In this section, I review the literature reporting associations of vitamin D and CVD and risk factors for CVD, and aim to assess whether the associations are causal using the Bradford Hill's criteria.¹⁰⁵

Observational associations of vitamin D with risk factors for cardiovascular disease

In 1990, Scragg et al first reported an association between low vitamin D levels and the risks of myocardial infarction in a small age and sex-matched case-control study (32 nmol/L vs. 35 nmol/L, $p=0.017$),¹⁰⁶ but these results were from a small sample size, and could be due to reverse causation. In addition, cross-sectional studies broadly demonstrate an inverse association of 25(OH)D levels with CVD risk factors.^{71, 107-111} In the third National Health and Nutrition Examination Survey (NHANES III)^{107, 111} of more than 13,000 people over the age of 20 living in the United States, plasma 25(OH)D levels were lower among those with established CVD risk factors such as smokers, those with hypertension, type 2 diabetes mellitus, obesity, impaired renal function, low albumin and in those with lower physical activity.¹⁰⁷ The plasma levels were also found to be inversely related with age, body mass index (BMI), total cholesterol and C-reactive protein (CRP).¹⁰⁷ Individuals in the lowest quartile of plasma 25(OH)D, had a 30% higher risk of hypertension (OR 1.30; 95% CI: 1.13 – 1.49), were twice likely to have high fasting glucose levels (OR 2.15; 95% CI: 1.69 – 2.74) or be obese (OR 2.29; 95% CI: 1.99 – 2.63), and more likely to have higher triglycerides (OR 1.47; 95% CI: 1.30 – 1.65).¹¹¹ These findings were similar in other prospective studies from Europe.^{64, 112}

Low vitamin D levels were also found to be associated with reduced cardiac contractility and lower concentrations of natriuretic peptides (peptides released as a response to myocardial stretch and often used as a surrogate for diagnosing heart failure).^{113 114, 115} Zittermann et al report that low 25(OH)D was more prevalent among patients with reduced left ventricular systolic function (and normal renal function) compared to controls.^{19, 114} Low levels were also seen in people with dark skin living in the Northern hemispheres, but whether these lower levels are responsible for the higher risks of CVD seen in these populations is unknown,^{71, 116-118} but could simply be the result of the migration of such populations, where the risks of CVD among the migrant people tend to match those of the hosts living in the locale.¹¹⁹

Arterial stiffness is an index of distensibility of the arterial wall (in simple terms), and is a surrogate marker for CVD¹²⁰ and has been associated with increased risks of CVD, independent of blood pressure.¹²¹⁻¹²³ Despite the differing indices and methods of estimation of arterial stiffness between studies,¹²⁴⁻¹²⁶ cross-sectional analyses show significant inverse associations of 25(OH)D levels with arterial stiffness measures.

Some prospective studies reporting baseline 25(OH)D concentrations showed baseline levels were predictive of the risks of incident hypertension^{110, 127, 128} and diabetes.¹²⁹⁻¹³² For instance, among the participants of the Health Professional Follow-up study and the Nurses' Health Study, a 3-fold higher risk of developing hypertension was seen among those who were vitamin D deficient (<32.5 nmol/L) at baseline compared to those who had sufficient concentrations (HR 3.18; 95% CI: 1.39 to 7.29).¹¹⁰ This association was independent of age, BMI, and level of physical activity. Low vitamin D levels also

predicted diabetes onset in a study of 9841 patients over 29 years, with those in the lowest quartile of 25(OH)D showing a 35% increased risk of developing type 2 diabetes (RR 1.35 95% CI:1.09-1.66) compared to those in the top quartile.¹³⁰

However, in the Scandinavian TROMSO study, baseline 25(OH)D did not predict incident hypertension over 14 years, although the concentrations of 25(OH)D correlated well with baseline measures of blood pressure.¹³³ Analysis of the participants in the Women's Health Initiative (WHI) trial of post-menopausal women also showed 25(OH)D levels were not associated with changes in blood pressure or incident hypertension after 7 years.¹³⁴ These discrepant results are difficult to interpret as baseline characteristics differ between the study participants recruited, and these differences on participant characteristics could affect other factors such as their motivation and adherence to treatment, geographic or climate factors, age and socio-economic status. For instance, the differences in the rates of use of concomitant treatment for blood pressure and diabetes control were different. Furthermore, the Health Professionals Study included predominantly male physicians living in the United States, the Nurses' Health study included women, the TROMSO study involved community-living participants in Norway, and the WHI trial recruited post-menopausal women. Hence, it is difficult to reconcile the differences between these study outcomes.

Only a limited number of studies reported on baseline 25(OH)D levels and incident CVD (by 2012); they often used varying definitions for CVD outcomes or used a composite outcome which included fatal and non-fatal events. In the Framingham Offspring study⁵⁹ of 1739 participants (mean age 59 years), low 25(OH)D levels at baseline were

associated with an increased risk of developing CVD events over 5.4 years. The 120 CVD events reported in the study included fatal and non-fatal myocardial infarction and strokes. The risk of CVD was higher in those with 25(OH)D levels <37.5 nmol/L (HR 1.62; 95% CI: 1.11-2.36), compared to those with 25(OH)D levels >37.5 nmol/L. In a much larger prospective analysis of 41504 electronic health records of the Intermountain Health Study registry,¹²⁸ an increased risk of incident CVD (this time a composite of death, coronary artery disease, heart failure, atrial fibrillation, stroke and peripheral vascular disease) was seen among those with baseline 25(OH)D levels <37.5 nmol/L, (adjusted HR 1.79; 95%CI: 1.53-2.10, $p < 0.0001$) compared to those with > 75 nmol/L. Low vitamin D levels were also shown to be predictive of the risks of developing heart failure, myocardial infarction and stroke.¹³⁵ In a European cohort of 548 patients with heart failure (aged 64–80 years), baseline 25(OH)D levels predicted death from any cause (HR 1.10 per 10 nmol/L decrease; 95% CI: 1.0–1.2).¹³⁶

However, these associations of low 25(OH)D levels with incident CVD are not consistent in all analyses.^{134, 137} Baseline 25(OH)D levels in older women participants of an Australian vitamin D supplementation trial were not found to be predictive of CVD outcomes.¹³⁷ However, these results may have been confounded by the inclusion of participants who received vitamin D supplements¹³⁷ and HRT.¹³⁴

The majority of prospective studies of vitamin D have evaluated mortality rather than incident events. A summary of the largest prospective studies (until Dec 2011) that have reported associations of baseline 25(OH)D with CVD or all-cause mortality are shown in

Table 2-1. Two previous meta-analyses found insufficient evidence on the associations

between 25(OH)D and CVD outcomes, but had fewer than 10 prospective studies in each of their analyses on CVD outcomes.^{138, 139}

Finally, though most studies report an inverse association, a 'curvilinear' or U-shaped association between 25(OH)D levels and the risks of mortality are reported in some studies.^{107, 140} Higher risks of mortality were observed among those with concentrations of 25(OH)D higher than 100-120 nmol/L at baseline. The nature of these associations was more apparent when participants were grouped in centiles of baseline 25(OH)D concentrations. However, these analyses had few participants with circulating 25(OH)D concentrations >120 nmol/L, to be able to explore these relationships with certainty.

Two large prospective studies have recently re-affirmed the possibility of a curvilinear or U-shaped relationship between vitamin D concentrations and the risks of all-cause mortality.^{141, 142} When individual participant data was evaluated from participants from multiple prospective study cohorts, the hazard ratios for all-cause mortality at > 140 nmol/L serum 25(OH)D was 1.42 (1.31-1.53) when compared to those with serum 25(OH)D of 50-60nmol/L.¹⁴¹ In the NHANES III population,¹⁴³ the lowest risks of mortality were seen at concentrations of about 90 nmol/L, but increased risks of mortality were observed at concentrations higher than 140 nmol/L. Chowdhury et al, noted that the inverse relationship with CVD deaths persisted through the range of plasma 25(OH)D distribution.¹⁴² However, in the CopD study, there were only 146 deaths (in over 3500 deaths) among participants with a 25(OH)D concentration >100 nmol/L in this study.¹⁴¹ The explanations for these higher risks of mortality at higher concentrations of 25(OH)D remain unclear. Although no significant hypercalcaemia

occurs at such concentrations of 25(OH)D, the higher mortality risks could be related to clinically non-apparent hypercalcaemia. Equally these may reflect outliers, who may be taking higher dose supplements after being recently ill. No specific assumptions could be drawn from the available data other than that further trials are necessary to evaluate whether higher plasma concentrations (>100-120 nmol/L) could be associated with an increased risk of dying from any cause.

The inverse associations of 25(OH)D with CVD risk factors reported in these cross-sectional analyses and prospective studies may also be due to reverse causation where those who are relatively ill are more likely to remain indoors compared to those who are otherwise healthy, and hence have lower 25(OH)D levels in blood. It could also be that low 25(OH)D is a marker of ill-health or frailty. Hence people who are obese and are smokers have lower levels, but they are more likely to be unhealthy and ill than others. Exploring the temporality of the association of baseline 25(OH)D levels with incident CVD, may provide further information (Chapter 3).

Possible mechanisms for low 25(OH)D causing CVD

The potential mechanisms by which low vitamin D levels could cause CVD are reviewed elsewhere.^{8, 45, 144, 145} Below is an overview of the key mechanisms (**Figure 2-3**) that have been proposed, but the effects of vitamin D on blood pressure and arterial stiffness is reviewed further, as this thesis aims to explore effects of vitamin D supplements on these markers of CVD.

The Renin-Angiotensin system

Vitamin D could affect the cardiovascular system through the actions on the renin-angiotensin-aldosterone system (RAS) and through its key effects on blood pressure and on cardiac muscle structure. In vitamin D receptor knockout (VDRKO) mice, activation of the RAS caused raised blood pressure and left ventricular hypertrophy.^{146, 147} When adequate levels of vitamin D are available, RAS is inhibited and blood pressure remains normal; when levels are low, there is reduced RAS inhibition. The active form of the vitamin D, 1,25(OH)D, may also directly inhibit RAS.¹⁴⁸ However, in humans with hereditary vitamin-D-resistant rickets, a rare genetic disorder where patients have resistance to vitamin D from birth, no activation of the RAS or evidence of cardiac muscle hypertrophy were found. In a case series published including follow-up of 17 cases, all patients with the disease had normal blood pressure. These findings therefore contradict those from the animal studies and indicate that the effects in human beings may differ as the pathophysiological pathways may be different between mice and man. Some of the observed inhibition of RAS in VDR KO mice may have been mediated indirectly through the changes in the PTH and FGF-23 pathways.

Effects of Parathyroid hormone or vitamin D

A chronically elevated PTH concentration has been shown to be independently associated with increased risks of CVD, including hypertension, cardiac hypertrophy and myocardial dysfunction.¹⁴⁹ Increased PTH levels were also associated with RAS activation and increased cardiac muscle hypertrophy. As elevated PTH levels can also occur in response to low circulating plasma 25(OH)D levels, some argue that PTH may

be an independent predictor of CVD when compared to low 25(OH)D levels;^{150, 151} however, as PTH concentrations appear to be mainly driven by the circulating concentrations of calcium in the body, and that calcium levels are influenced by a number of other factors, it seems unlikely that hyperparathyroidism alone could explain or account for a significant proportion of CVD. In contrast, circulating 25(OH)D levels are influenced by a number of external and internal factors, has a broader role in pathophysiology and actions through a number of potential mechanisms that could influence the development of CVD. Finally, VDR down regulates the parathyroid hormone and the enzyme CYP27B1,¹⁵² and hence possibly has the higher control function in the calcium-vitamin D metabolism pathway.

Atherosclerosis

The development of atherosclerosis is predominantly an inflammatory process.¹⁵³ There is evidence demonstrating a key role for vitamin D in the regulation of inflammatory and immune responses and is extensively reviewed elsewhere.^{7, 41, 45} The presence of VDR is now recognised in most immune and inflammatory cells. The metabolism of vitamin D (hydroxylation into active calcitriol and its breakdown to other inactive metabolites) is locally regulated in these tissues and appear to correlate well with the local needs and the circulating levels of 25(OH)D.

There is evidence from animal models suggesting that low blood concentrations of vitamin D promotes atherosclerosis by the up-regulation of macrophage cholesterol uptake, worsening endothelial dysfunction, increased vascular smooth muscle cell proliferation and migration, endothelial activation and increased expression of

endothelial adhesion molecules.^{45, 154} In the presence of low vitamin D levels, anti-inflammatory processes are inhibited and hence chronic inflammation continues unabated promoting CAD.⁴⁵ VDR knockout mice exhibit up-regulation of metalloproteinases (enzymes linked to cardiac repair after injury). Adequate vitamin D may help promote anti-inflammatory processes, through effects mediated via interleukins, C-reactive protein, and anti-inflammatory cytokines such as IL-10. Adequate vitamin D inhibits IL-6 and TNF-alpha.⁷ By keeping a balance between the pro and anti-inflammatory processes, adequate vitamin D may keep atherosclerosis under control. In summary, in animal studies low vitamin D appears to promote a pro-inflammatory milieu that may help drive pro-atherosclerotic processes in those with CVD risk factors and is evidence for the 'paracrine' function of vitamin D.

Effects on calcium handling and cardiac function

Another possible mechanism for the effects of vitamin D on CVD is through the effects on cardiac function such as contractility and automaticity, which are mediated through calcium ions. Vitamin D influences myocardial calcium metabolism in rats where calcium metabolism in the cardiac muscle is mediated by a vitamin-D dependant receptor.¹⁵⁵ Similarly, vitamin D deficient rats have increased collagen content in the myocardial tissue demonstrate higher blood pressure, cardiac muscle hypertrophy and cardiac fibrosis compared to rats with sufficient levels of vitamin D. Cardiac contractility may also be mediated as a result of the rapid response of the membrane based VDR binding of calcitriol.¹⁵⁶ Vitamin D could influence cardiac muscle relaxation and indirectly the intracellular calcium influx by its effects on other pathways.⁴⁵

Observational data also show associations between low 25(OH)D and reduced cardiac contractile function perhaps through the abnormal calcium handling,¹⁵⁷ as low vitamin D levels may cause a dysregulation in the metabolism of these ions leading to abnormal cardiac function. The VDRKO mice exhibit impaired cardiac function (both relaxation and contraction).¹⁵⁸ Children with rickets are known to develop cardiac failure which is reversible once vitamin D sufficiency is restored.¹⁵⁹ Although there is no conclusive evidence for the effects of taking vitamin D on cardiac function, administration of vitamin D supplements was associated with a reduction in the pro-inflammatory cytokines among patients with heart failure.¹⁶⁰

Arterial stiffness

Arterial stiffness is associated with the risks of developing CVD. Increased stiffness is an indicator of biological aging and is seen more commonly among older people, those with CVD risk factors and those with established atherosclerosis.¹⁶¹ There are different theories as to why the vessels become stiffer, such as the stiffening of arterial walls with repeated mechanical stress, damage to the collagen fibres in the walls, the increased fibrosis as a result of cumulative damage or the increased calcification of the vessel walls. Further, the effects of low vitamin D concentrations on the arterial wall may be different from circulating levels as the cells such as the vascular smooth muscle and endothelial cells, and the macrophages produce active calcitriol locally.¹⁶²

Observational studies suggest that low vitamin D is associated with increased arterial stiffness.^{124, 126, 163} High vitamin D in humans, is associated with increased calcification mainly in the setting of chronic renal failure causing calcium deposits in the body,¹⁶⁴ but

animal models suggest that low vitamin D levels could also promote vascular calcification.¹⁶² In mice with chronic kidney disease, supplementation of vitamin D sufficient to suppress PTH caused a reduction in the expression of osteoblasts in the aorta, but high doses caused calcification.¹⁶⁵ The Klotho gene associated with premature aging in mice and humans is believed to influence vascular calcification along with influences from vitamin D and FGF-23.¹⁶⁶ Klotho deficient mice show accelerated atherosclerosis and increased vascular calcification. In patients with chronic renal failure, low vitamin D and calcification are commonly associated. Hence the mobilisation of calcium in the presence of low vitamin D increases vessel wall calcification.^{162, 164}

Alternatively, the increased arterial stiffness in the presence of low vitamin D may be a result of cumulative vessel wall damage mediated by the effects on other co-existing CVD risk factors. Whether arterial stiffness is modifiable by administering vitamin D supplements is unknown and so evaluating the effects of vitamin D supplements on arterial stiffness could offer insights into potential mechanisms of vitamin D on CVD outcomes before randomised trials of vitamin D provide evidence on CVD endpoints.

Evidence from randomised trials

Effects on CVD risk factors

Randomised trials of vitamin D have failed to confirm any beneficial effects of taking dietary supplementation of vitamin D on any of the CVD risk factors.^{139, 167} However, most trials of vitamin D supplements were conducted among the elderly and focussed

mostly on bone outcomes such as fractures and falls. There is only limited data available from the previous vitamin D trials of CVD outcomes, and the meta-analyses,^{139, 167-169} of such randomised trials reported no effects for taking vitamin D supplements on blood lipids, glucose or blood pressure (discussed further below). So far, no studies have shown consistent effects of taking vitamin D supplements on markers of inflammation, endothelial function, CRP, renal function or diabetes. The authors however highlighted the paucity of reliable good quality trial data to address these questions,^{139, 167, 170} and there is a call for further trials.¹⁷¹

There has been particular interest in the effects of vitamin D on blood pressure, given the studies in animals.¹⁴⁸ One of the first vitamin D trials in humans to demonstrate blood pressure lowering, 148 older women (mean age 74) taking vitamin D (800 IU) with calcium (1200 mg) daily had significantly lower systolic blood pressure (reduced by 9.3%) after 8 weeks, compared to those taking calcium alone.¹⁷² However, there is no consistent evidence from randomised-controlled trials that taking vitamin D3 supplements lowers blood pressure.^{139, 173-177}

In an earlier meta-analysis¹⁷⁸ of vitamin D trials and blood pressure lowering (11 trials, n = 716), the authors evaluated 8 trials where participants had mean baseline blood pressures >140/90 mm Hg, and found a non-significant reduction in systolic blood pressure [-3.6 mmHg, 95% CI: -8.0 to 0.7] and a significant reduction in diastolic blood pressure [-3.1 mmHg, 95% CI: -5.5 to -0.6] among those allocated to vitamin D. However, conflicting results were observed in a subsequent meta-analysis¹³⁹ of 10 randomised trials of vitamin D (9 trials using vitamin D supplements and one trial of

UVB treatment, n=37162), where no evidence of reduction in systolic or diastolic blood pressures (mean difference of -1.9 mm Hg for SBP; 95% CI: -4.2 to 0.4 mm Hg) were found. Only 2 trials in this analysis reported large effects in reducing blood pressure,^{172,179} but the largest number of participants (98%, n = 36282) contributing to these analyses were data from the Women's Health Initiative (WHI) trial of post-menopausal women examining the effect of taking 400 IU/d of cholecalciferol (with 1000 mg/d of calcium) versus placebo, on the incidence of fractures and cancer in post-menopausal women.¹⁸⁰ After 7 years of follow-up no significant differences in mean levels of blood pressure were detected between the vitamin D and placebo allocated groups, or among women with hypertension, among the black participants, or among those who had lower vitamin D at randomisation. However, this trial may not be ideal to evaluate effects on CVD (discussed later) as the doses used were small and insufficient to achieve or maintain the 'optimal levels' of 25(OH)D. Hence, there is a substantial influence from this large trial with negative findings for vitamin D supplementation on this and other meta-analyses of the randomised controlled trials of vitamin D, which is important when contextualising the neutral results so far, for taking vitamin D supplements on various CVD outcomes.

Three recent trials testing the effects of much higher doses of vitamin D supplements on blood pressure were recently published, but reported conflicting results. The VitDish trial¹⁷⁷ used 3-monthly doses of 100 000 IU cholecalciferol or matching placebo, in 159 Scottish participants with isolated systolic hypertension (mean age 77 years). No differences in blood pressure were found at the end of 1 year [mean baseline 25(OH)D was 45 nmol/L]. A Norwegian trial recruited 438 overweight and obese people (age 21 –

70 years old), and randomised participants to receive 40 000 IU or 20 000 IU of vitamin D3 weekly or placebo.¹⁸¹ The mean baseline 25(OH)D levels were higher (58 nmol/L) and did not show any effects on blood pressure lowering after 1 year. However, in another trial of 283 African-American participants taking 2000 IU or 4000 IU vitamin D3 daily or placebo,¹⁸² a significant reduction in systolic blood pressure of 1.4mm Hg with every 2.5 nmol/L rise in 25(OH)D levels was noted, after 3 months of treatment.

These contrasting findings are difficult to interpret in the first instance, given the differences in the trial participant characteristics, the nature of the participants (hypertensive people, obese, African Americans) and the differences in the duration of treatment. However, the findings broadly indicate that the effects of vitamin D supplementation on blood pressure needs testing in larger trials, as there are at least some trials that show beneficial effects for taking vitamin D, and the reasons for the disparity may either reflect the inadequate sample sizes of the individual trials or the need for further participant characterisation to understand who would be suitable for vitamin D therapy; but the latter approach may be logistically more difficult. The meta-analyses of vitamin D trials have not shown consistent benefits, but many included trials that used insufficient doses of vitamin D such as the WHI trial. Whether treatment with vitamin D supplements would be more effective with higher doses of supplements, or after a longer duration of treatment is unclear. The observational literature would support the hypothesis that the benefits to be had are greatest when a doubling from baseline concentrations of 25(OH)D could be achieved.

Since the thesis submission, a meta-analysis of 46 trials which included individual patient data from 27 trials (3092 patients),¹⁸³ was published, and found no differences in blood pressure lowering after taking vitamin D3 supplements. The analysis also failed to identify sub-groups that could benefit from supplementation, when stratifying by baseline 25(OH)D levels, blood pressure or presence of diabetes. However, the analysis included trials that used any form of vitamin D supplement (vitamin D2, vitamin D3 or analogues), and the vast majority of participants with patient-level data had normal blood pressure at baseline, which may have attenuated any possible effects of blood pressure lowering given the healthier profile, and the smaller numbers. In the analysis of patient-level data (about 1500 participants on vitamin D treatment), there was a non-significant lowering of systolic blood pressure among those taking vitamin D (effect size, -0.5; 95% CI: -1.3 to 0.4 mmHg).

Effects on arterial stiffness

Few randomised trials of vitamin D supplements have evaluated the effects on arterial stiffness and **Table 2-2** shows a summary of the trials with at least 3 months or more of follow-up (until 2014). These published reports typically included small numbers of participants, had relatively short follow-up periods and used varying methods and indices for estimating arterial stiffness, and hence difficult to compare. Preliminary overall results indicate vitamin D has no effects on arterial stiffness. However, 2 trials reported improvements in arterial stiffness measures after vitamin D supplementation. In a study of young African teenagers, a daily intake of 2000 IU daily of vitamin D had showed a reduction in arterial stiffness after 4 months.¹⁸⁴ In a Danish study of patients with hypertension,¹⁷⁵ intake of 3000 IU daily over a 5 month period in winter was

associated with a reduction in central artery stiffness, but with no changes in the ambulatory blood pressure. Alternatively, it could mean that vitamin D does not have significant detectable effects on arterial stiffness, similar to the analyses of the effects on blood pressure. The effects of vitamin D on arterial stiffness need further testing in a large population and for a longer duration (ensuring that patient characteristics such as baseline blood pressure and concomitant treatment for blood pressure are taken into account in the analysis) before conclusions on any potential effects or the lack of it may be made.

Effects on CVD

No trials of vitamin D have so far been conducted to evaluate CVD as a primary outcome. Meta-analyses of results from randomised-controlled trials of vitamin D conducted so far have reported no beneficial effects of taking vitamin D on CVD outcomes including CVD deaths.^{139, 167, 170, 185} However, there is a lack of good quality evidence from the previous randomised trials of vitamin D on CVD outcomes. The baseline characteristics, the vitamin D supplement doses used and the numbers of events in the randomised groups in the largest trials of vitamin D, (with at least 1000 participants each) are shown in **Table 2-3** (adapted from meta-analyses.^{167, 170}

One of the recent analyses included around 40,000 patients,¹⁸⁵ involving 4 trials reporting CVD outcomes, 6 trials on myocardial infarction and 7 trials on CVD mortality and reported no effects on CVD. The typical daily dose of vitamin D ranged between 320 IU to 1400 IU in the trials included and the WHI trial contributed most to the analysis. Most previous trials of vitamin D supplements were primarily designed to evaluate effects on falls and fractures in older people, and not CVD outcomes.

The WHI trial¹⁸⁰ the largest trial of vitamin D to date, contributes most to the evidence presented in the above meta-analyses on the effects of vitamin D on CVD outcomes. This trial included 36282 post-menopausal women who were allocated to vitamin D3 200 IU twice daily (with calcium) or placebo and found no differences on CVD outcomes after 7 years (HR, 1.04; 95% CI: 0.92 to 1.18). The dose of vitamin D used in this trial¹⁸⁰ (400 IU daily) would have been sufficient to increase 25(OH)D levels only by about 7-10 nmol/L.¹⁸⁶ Given these factors, a significant difference in circulating levels of 25(OH)D between treatment and comparison groups [blood 25(OH)D was not measured at end of trial; participants were permitted open-label vitamin D supplements also] would not have been achieved at the end of the trial and hence it is unlikely that the trial could have identified any meaningful differences in CVD outcomes despite the long follow-up period. However, until further trials using higher vitamin D doses or enrolling large enough number of participants become available, the meta-analyses of trials of vitamin D and assumptions based on their findings will continue to be influenced by the null results of the WHI trial.¹⁸⁷

Effects on all-cause mortality

The available evidence on the effects of vitamin D supplements on risks of death is based on a number of published meta-analyses evaluating the previous trials. The first meta-analysis¹⁸⁸ included published data from 18 randomised trials of vitamin D alone or with calcium supplements, and showed a 7% reduced risk of death [Relative risk (RR) 0.93; 95% CI: 0.87 – 0.99] for those taking vitamin D supplements. However, there were only 2 un-confounded trials of vitamin D in this analysis (other trials used vitamin D and calcium supplements).

The larger of the two unconfounded trials,¹⁸⁹ recruited community dwelling older people in the UK, where participants received either 100,000 IU (equivalent to 800 IU daily) or placebo every 4 months. This trial reported a non-significant difference in all-cause mortality (RR 0.88; 95% CI: 0.74 to 1.06), and in deaths due to CVD (RR 0.84; 95% CI: 0.65 to 1.10) among those taking vitamin D.

Other meta-analyses^{142, 190, 191} of vitamin D supplement trials since published have broadly reported consistent trends in reduced mortality among those taking vitamin D supplements. However, in one analysis which included patient-level data from over 70,000 patients, a trend towards benefit of lower mortality risks was noted when calcium was administered with vitamin D but not when vitamin D supplements were given alone.¹⁹¹ Another analysis that included data from 22 vitamin D trials showed a reduction in mortality with taking vitamin D3 supplements only but not for taking vitamin D2.¹⁴²

It is unclear whether or how the calcium supplementation could have influenced the findings in these analyses of vitamin D trials, as the use of calcium supplements was found to be associated with increased risks of myocardial infarction.¹⁹² Calcium intake significantly increased the risks of myocardial infarction by 31% (HR 1.31; 95% CI: 1.02-1.67, p=0.035) when patient-level data from five trials and 8151 participants were examined. It is possible that concomitant intake of vitamin D and calcium could obscure the apparent benefits of taking vitamin D alone, but it is contrary to the findings from Reinmark et al.¹⁹² Furthermore, the mortality benefits may only be associated with vitamin D3¹⁴² intake and including data from trials using vitamin D2 could have led to

the discrepant findings. It may also mean that the mortality benefits due to calcium and vitamin D intake are greater than the risks of myocardial infarction associated with calcium supplements; hence for non-cardiovascular causes of mortality, taking vitamin D and calcium maybe beneficial. One other possible explanation could be that the additive effects of taking vitamin D and calcium could help normalise the parathyroid hormone levels more effectively (PTH is an independent risk factor for mortality).^{149, 150} Given the evidence from genetic studies of possible mortality benefits with higher vitamin D status,¹⁹³ a further trial using treatment groups taking vitamin D alone, and with concomitant calcium, could provide answers. A small number of trials using higher doses of vitamin D are already underway (See **Table 2-4**) to evaluate effects on a number of health outcomes including CVD and all-cause mortality, but the earliest trial results assessing effects on CVD outcomes are not expected until 2017.

Vitamin D and CVD: Mendelian randomisation studies

Few published Mendelian randomisation studies have examined the effects of vitamin D on CVD outcomes (**Table 2-5**). One of the recent studies¹⁹⁴ examined the associations of vitamin D status and the risks of hypertension and reported increased 25(OH)D levels to be associated with a reduced risk for hypertension. For each 25(OH)D allele added to the model, a 0.1 mm reduction in systolic blood pressure (95% CI: -0.21 to -0.0001; $p=0.0498$) was noted and a decrease of 0.08mm Hg diastolic blood pressure (95% CI: -0.15 to -0.02; $p=0.01$). These findings will need further evaluation as the evidence from randomised clinical trials show no effects for taking vitamin D supplements on blood pressure.¹⁸³

However, given the biology of the vitamin D and the range of actions linked with the VDR activation, evidence from Mendelian randomisation studies of vitamin D should also be interpreted with caution to avoid erroneous attribution of causality. One of the other major constraints for Mendelian studies of vitamin D is identifying suitable single nucleotide polymorphisms (SNPs) or alleles, that translate into large enough differences in circulating concentrations of 25(OH)D. In one evaluation for suitable SNPs for vitamin D status, the 'per allele difference' in plasma levels of 25(OH)D were only between 0.02 to 0.08 nmol/L (roughly equivalent to the effect of taking 3.2 IU of vitamin D3 daily), and when adding up the alleles, the highest number of risk alleles translated to a difference of only about 10 nmol/L (roughly equivalent to taking 400 IU of vitamin D3 daily).¹⁹⁵

Finally, the population cohort studies also need to consider population admixture and the effects of it, as people living in geographic locations with low UVB availability for generations may have adapted already to counter the adverse effects of low vitamin D on normal physiology, and this could attenuate any causal effects.⁴⁶

Why further prospective studies of 25(OH)D and CVD might be informative

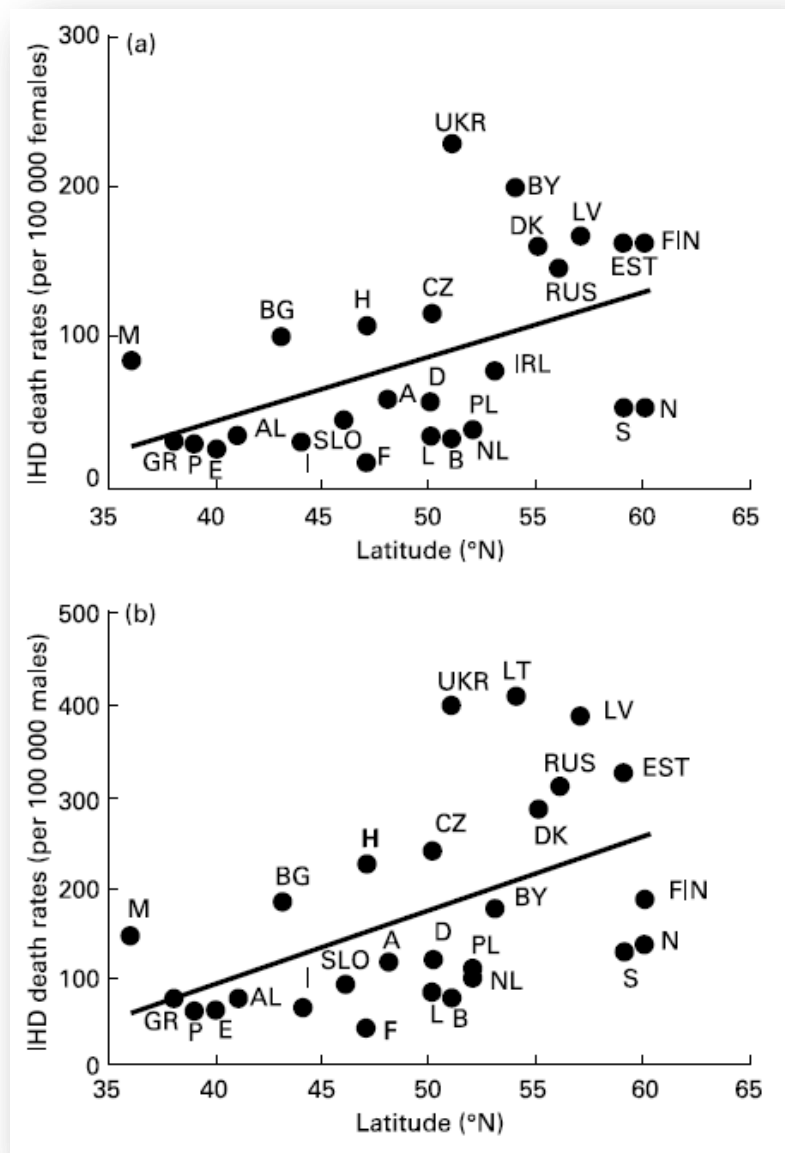
There is a need to evaluate the shape and strength of the associations of baseline 25(OH)D and CVD deaths in a larger prospective study. Although the preliminary reports from prospective studies of 25(OH)D broadly confirm inverse associations with CVD risk factors and CVD, factors such as reverse causation, the links with reduced physical activity and residual confounding need further exploration.

The strength and consistency of the reported associations between 25(OH)D and CVD outcomes need to be established. The approach in evaluating associations need to be more consistent; for instance, in methods used for the correction of seasonal adjustment for measured 25(OH)D levels, in applying methods to evaluate and minimise the effects of reverse causality on any risks identified, and using appropriate variables in the regression models. Further, the plasma 25(OH)D levels conferring the lowest risks for CVD mortality remains un-established; it is unclear if higher plasma levels of 25(OH)D could be harmful. Finally, previous meta-analyses of prospective studies of 25(OH)D have highlighted the paucity of good quality studies, to provide reliable information and hence the need for a further study.

Conclusion

In this chapter, we reviewed the published evidence from observational studies of vitamin D and CVD, and evaluated the evidence from randomised trials and Mendelian studies of vitamin D on CVD and related outcomes. The evidence from these previous studies remains inconclusive and further studies are needed to clarify if there is a causal role for low vitamin D for prevention of CVD. In the next chapter, the shape and strength of the associations between plasma 25(OH)D and CVD and cause-specific mortality are evaluated.

Chapter 2: Figures and Tables

Figure 2-1: Associations between geographic latitude and IHD rates in Europe(adapted from Zittermann et al⁸)

(a) females (r 0.49; P < 0.01) and (b) males (r 0.51; P < 0.01) of different European countries. A, Austria; AL, Albania; B, Belgium; BG, Bulgaria; BY, Belarus; CZ, Czech; D, Germany; DK, Denmark; E, Spain; EST, Estonia; F, France; FIN, Finland; GR, Greece; H, Hungary; I, Italy; L, Luxembourg; LT, Lithuania; LV, Latvia; M, Malta; N, Norway; NL, Netherlands; P, Portugal; PL, Poland; S, Sweden; SLO, Slovenia; RUS, Russia; UKR, Ukraine.

Figure 2-2: Overview of vitamin D and calcium metabolism

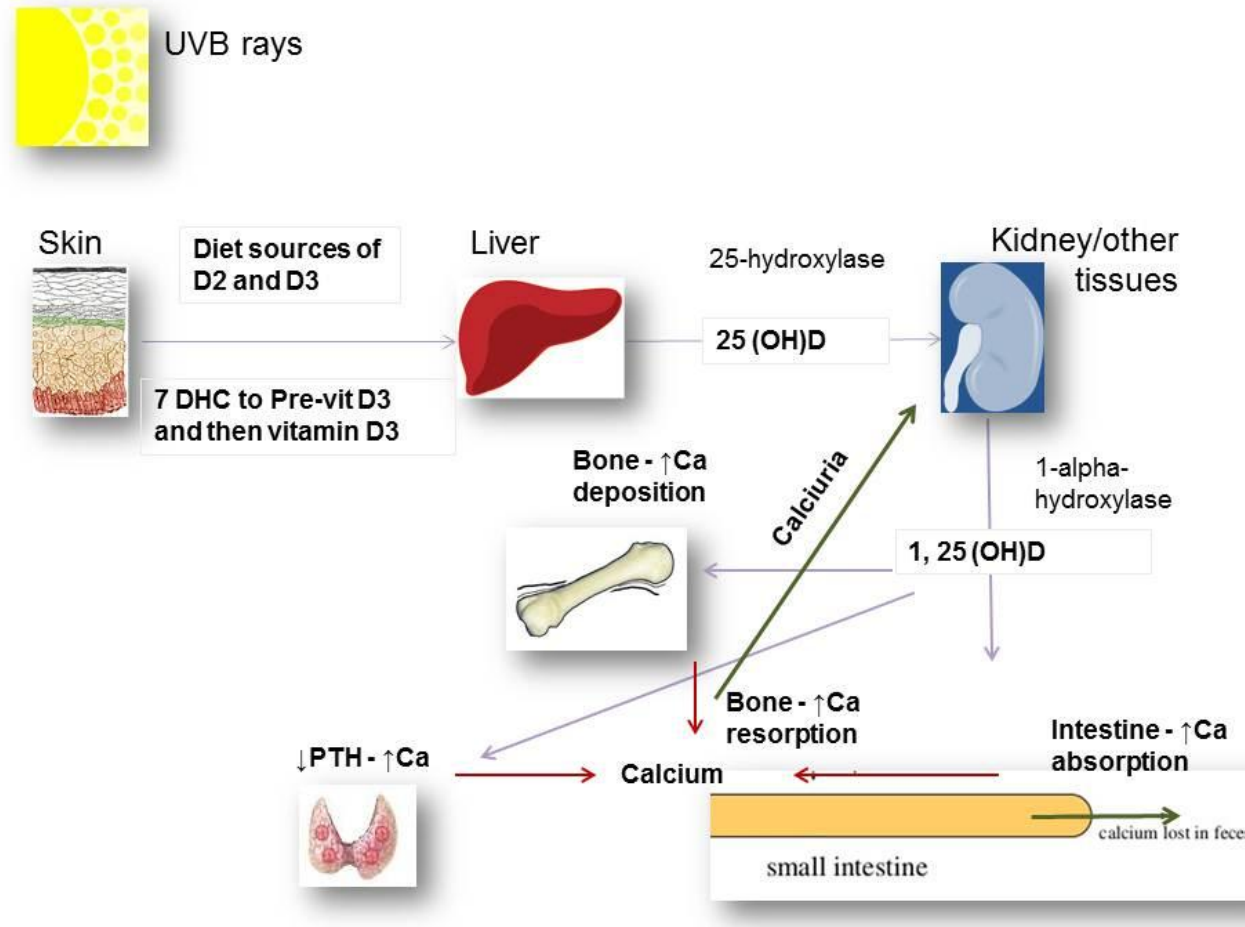
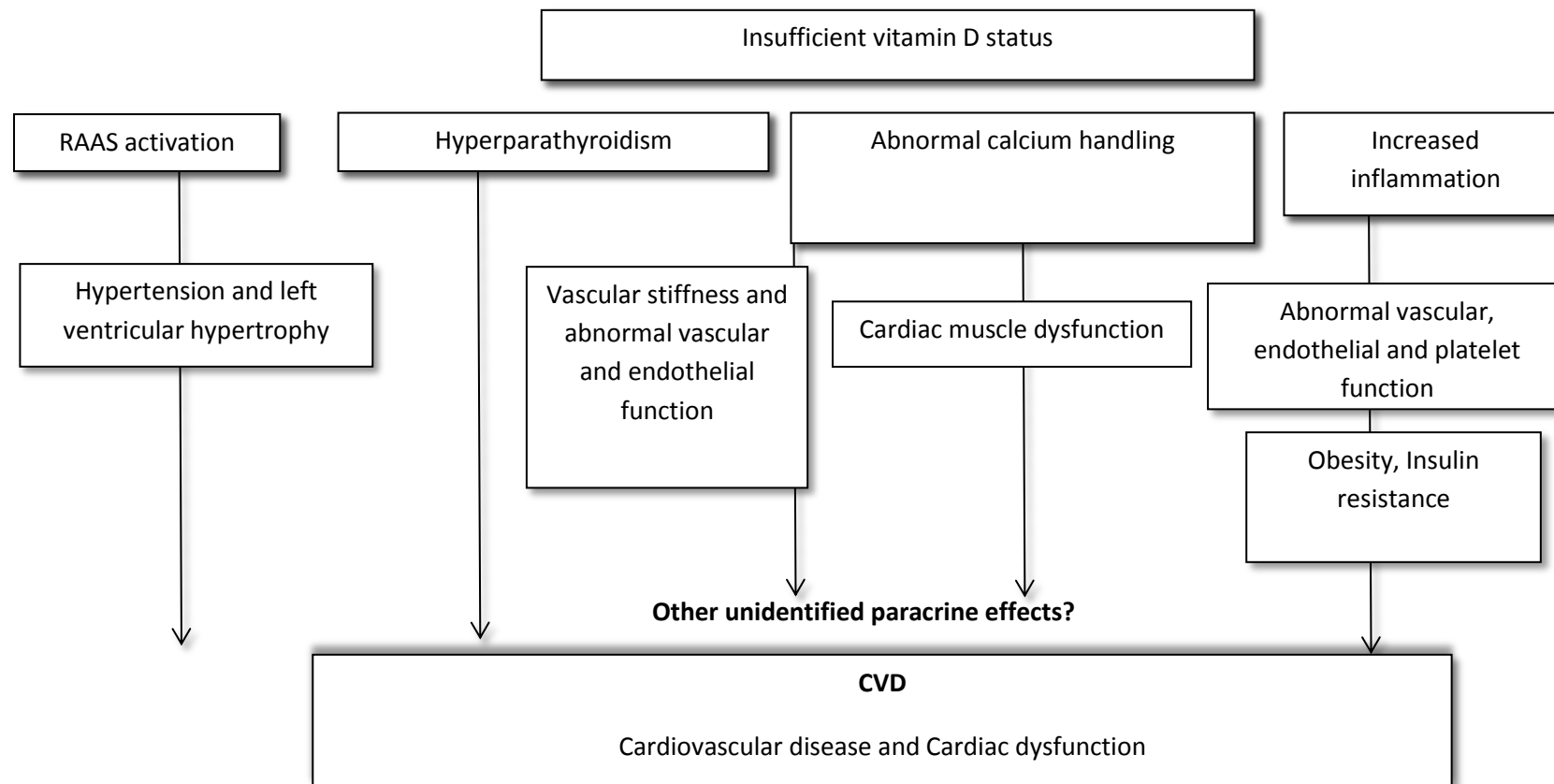


Figure 2-3: Potential mechanisms how low vitamin D causes cardiovascular disease



RAAS, renin-angiotensin-aldosterone system; CVD: cardiovascular disease;

Table 2-1: Prospective studies reporting on associations of baseline 25(OH)D and risks of CVD and all-cause mortality

(Updated 2013)

| Author | Year | Country | Characteristics | Follow-up (Yrs)* | Participants (no.) | Males (%) | Risk factors in fully adjusted regression** |
|---------------------------|------|-----------|---|------------------|--------------------|-----------|---|
| Anderson ¹²⁸ | 2010 | US | Evaluation of the electronic medical records database of the Intermountain Healthcare system. | 1.3* | 27686 | 25 | 1347 |
| Bolland ¹³⁷ | 2010 | Australia | Women participants in a randomised trial; (78 women were treated with 10,000 IU of vitamin D3 for 10 days). | 5 | 1471 | 0 | 12 |
| Cawthon ¹⁹⁶ | 2010 | US | Prospective osteoporotic fractures in Men (MrOS study) ; men >65 yrs. | 7.3 | 1490 | 100 | 125 |
| Dobnig ¹⁹⁷ | 2008 | Germany | Patients referred for coronary angiography in the -The Ludwigshafen Risk and Cardiovascular Health (LURIC) study. | 7.7 | 3258 | | 13467 |
| Ensrud ¹⁹⁸ | 2010 | US | Post-menopausal women >69 years. | 4.5 | 4551 | 0 | 128 |
| Fiscella ⁷¹ | 2010 | US | The National Health and Nutrition Examination Survey (NHANES). | 9 | 15363 | 48 | Ns |
| Ford ¹⁹⁹ | 2011 | US | Participants > 17 years in the National Health and Nutrition Examination Survey III (NHANES III) Mortality Study. | 3.8 | 7531 | 51 | 1234578 |
| Ginde ²⁰⁰ | 2009 | US | Participants in the NHANES III survey who were > 65 years. | 7.3* | 3408 | 98 | 1234578 |
| Giovanucci ¹³⁵ | 2008 | US | Health professionals study. Nested case - control study | 10 | 900 | 100 | 123467 |

Vitamin D and Cardiovascular disease

| | | | | | | | |
|----------------------------|------|---------|--|-------|-------|-----|-------------------|
| | | | of men aged 40-75 years. | | | | |
| Grandi ²⁰¹ | 2010 | Germany | KAROLA study- (Langzeiterfolge der KARdiOLogischen); patients recruited from 2 German cardiac rehab clinics. | 8 | 1125 | 85 | 1234679 |
| Hutchinson ¹³³ | 2010 | Norway | Follow-up of TROMSO 4 study participants. | 11.7 | 7161 | | 137, prior cvd |
| Jassal ²⁰² | 2010 | US | Adults living in Southern California - The Ranch Bernardo study. | 6.4 | 1073 | 74 | 123467 |
| Jia ²⁰³ | 2007 | UK | People > 75 years living in Aberdeen. | 5.7 | 399 | 52 | 12678 |
| Joergensen ¹³⁰ | 2010 | Denmark | Patients with type 2 diabetes < 66 years recruited from the Hvidovre hospital. | 15 | 289 | 58 | 1347 |
| Kitamura ²⁰⁴ | 2010 | Japan | Follow-up of 205 community-dwelling elderly people in the Yamato study. | 2 | 205 | 31 | NS |
| Kilkinen ¹¹² | 2009 | Finland | The Mini-Finland Health survey. Men and women >= 30years who were free of CVD at enrolment. | 27.1 | 6219 | 45 | 123 |
| Kuroda ²⁰⁵ | 2009 | Japan | Japanese postmenopausal ambulatory women. | 6.9 | 1232 | 0 | 1 |
| Marniemi ²⁰⁶ | 2005 | Finland | Case-control study of 65-99 year old people living in Turku chosen from Social insurance register. | 10 | 765 | 47 | 1 |
| Melamed ¹⁰⁷ | 2008 | US | Participants of the Third National Health and Nutrition Examination Survey (NHANES III); Participants >20years; 32% had prior CVD. | 8.7 | 13331 | Ns | 12347 9 |
| Michaelsson ¹⁴⁰ | 2010 | Sweden | Uppsala Longitudinal Study of Adult Men (ULSAM) study; elderly men living in the community; recruited 1991 – 1995. | 12.7* | 1194 | 100 | 1234678 |
| Pilz ¹¹⁴ | 2008 | | Patients referred for coronary angiography in the Ludwigshafen Risk and Cardiovascular health (LURIC) | 7.7* | 3316 | - | 1347 |

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| | | | study. | | | | |
|---------------------------|------|-------------|---|-----|------|-----|----------|
| Pilz ²⁰⁷ | 2009 | Finland | Hoorn study of older men and women; recruited 2000-2001. | 6.2 | 614 | ~50 | 1347 |
| Sambrook ²⁰⁸ | 2006 | Australia | The Fracture Risk Epidemiology in the frail Elderly (FREE) Study of men and women living in residential care. | 2.2 | 1112 | 21 | ns |
| Sambrook ²⁰⁹ | 2004 | Australia | Frail Older people living in nursing homes -FREE study. | 2.6 | 842 | 182 | ns |
| Semba ²¹⁰ | 2010 | Italy | People>65 years living in InCHIANTI. | 6.5 | 1006 | - | 12846 |
| Semba ²¹¹ | 2009 | US | Womens health and aging studies I and II; older women living in the community. | 6 | 714 | 0 | ns |
| Szulc ²¹² | 2009 | France | MINOS study of men > 50 years. | 10 | 782 | 100 | 18 |
| Visser ²¹³ | 2006 | Netherlands | Longitudinal Aging study Amsterdam (LASA); participants >65yrs of age. | 6 | 1260 | ~50 | 148 |
| Virtanen ²¹⁴ | 2010 | Finland | Kuopio IHD risk factor study (KIHD) - men and women free of CVD or cancer at baseline | 9.1 | 1136 | ~50 | 123748 |
| Wang ⁵⁹ | 2008 | US | Framingham Offspring study; participants free of CVD at baseline. | 5.4 | 1739 | 45 | 13749 |
| Tomson ⁹ | 2013 | UK | Resurvey of Whitehall study participants | 13 | 5409 | 100 | 12346789 |
| Zittermann ²¹⁵ | 2009 | Germany | Participants recruited from hospital (67% with HF, 33.7% with CAD); | 1 | 510 | - | 1379 |

Legend: *Indicates median follow-up. **Adjustment model codes: Each of the factors accounted for in the regression models used in the studies were attributed number codes as follows: Age and/or sex = 1; Season = 2; Hypertension = 3; Cholesterol or use of lipid-modifying drugs = 4; Race = 5; Medication use = 6; Diabetes or use of diabetic medications = 7; Socio-economic /educational status = 8 ; C-reactive protein = 9

Table 2-2: Randomised trials of vitamin D and arterial stiffness (with at least 3 months treatment)

(updated July 2014)

| Author | Mean age | Number (n) | Study characteristics | Vitamin D dose, type and frequency | Device | Follow-up (months) | Outcomes |
|---------------------------|----------|------------|---|--|---|--------------------|---------------------------------|
| Mose ²¹⁶ | 68 | 64 | Chronic renal failure patients on dialysis | 3000 IU oral vitamin D3 daily | PWV, AIX, central BP;ns | 6 | No change in arterial stiffness |
| Witham ²¹⁷ | 41 | 50 | Healthy South Asian women living in UK with 25(OH)D <75 nmol/L | Single dose of 100,000 IU oral vitamin D3 | Carotid-radial PWV and AIX; Sphygmocor. | 2 | No change in arterial stiffness |
| Witham ²¹⁸ | 77 | 159 | Patients 70 years or older with isolated systolic hypertension | 100,000 IU oral vitamin D3 every 3 months | Carotid-radial PWV and AIX; Sphygmocor. | 12 | No change in arterial stiffness |
| Breslavsky ²¹⁹ | 66 | 47 | Patients with type 2 diabetes | 1000 IU oral vitamin D3 daily | Carotid-radial PWV and AIX; Sphygmocor. | 12 | Improvement in AIX after 1 year |
| Hewitt ²²⁰ | 62* | 60 | Chronic renal failure patients on dialysis with 25(OH)D < 60 nmol/L | 50,000 IU oral vitamin D3 once weekly for 8 weeks; then monthly for 4 months | PWV | 6 | No change in arterial stiffness |

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|-----------------------|----|------|---|--------------------------------|-----------------------------|---|--|
| Larsen ¹⁷⁵ | 61 | 112† | Danish patients with hypertension recruited during winter | 3,000 IU oral vitamin D3 daily | Carotid-femoral PWV or Aix. | 5 | No change in arterial stiffness. Reduction in central blood pressure |
| Gepner ²²¹ | 64 | 114 | Post-menopausal women with 25(OH)D >25 and <150 nmol/L. | 2500 IU oral vitamin D3 daily | Carotid-femoral PWV and Aix | 4 | No change in arterial stiffness |
| Dong ²²² | 16 | 49 | Healthy black teenagers | 2000 IU oral vitamin D3 daily | Carotid-femoral PWV and Aix | 4 | Modification of Aix, but not PWV after 4 months |

Legend: * Median; † refers to the number of participants included in the (on treatment analysis); Aix = Augmentation index; PWV = Pulse wave velocity; ns – not specified.

Table 2-3: Largest randomised trials of vitamin D and CVD outcomes*

(updated August 2014)

| Author, Year | Mean age(yrs) | N | Participant characteristics | Vitamin D dose | Duration (months) | Primary outcome | Other outcomes | Vitamin D arm (Events / total participants) | Placebo arm (Events / total participants) |
|------------------------------|---------------|------|--|--|-------------------|-----------------------------------|------------------------------|---|---|
| Chapuy, ²²³ 1992 | 84 | 3270 | French women living in institutions | Daily oral vitamin D3 (800 IU)+ calcium (1.2 g) | 18 | Fractures | Death | 258/1634 | 274/1636 |
| Lips, ²²⁴ 1996 | 80 | 2578 | Dutch women living in institutions | Daily oral vitamin D3 (400 IU) | 42 | Fractures | Death | 223/1291 | 251/1287 |
| Meyer, ²²⁵ 2002 | 85 | 1144 | Norwegian older people living in institutions | Daily oral cod liver oil, more or less vitamin D3 (400 IU) | 24 | Fractures | Death | 169/569 | 163/575 |
| Trivedi, ¹⁸⁹ 2003 | 75 | 2686 | Older men and women living in the community in Oxfordshire | Oral vitamin D3 (100 000 IU every 4 months) | 60 | Fractures and all-cause mortality | MI or IHD Stroke Death | 224/1345 105/1345 224/1345 | 233/1341 101/1341 247/1341 |

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|---|----|-----------|---|---|----|--|--|---|---|
| Porthouse, ²²⁶ 2005 | 77 | 3314 | Women at risk of hip fracture living in the community | Daily oral vitamin D3(800 IU)_calcium (1 g) | 36 | Fractures | Death | 57/1321 | 68/1993 |
| Grant, ²²⁷ 2005 | 77 | 5292 | Older people with previous fracture living in the community (UK) | Daily oral vitamin D3 (800 IU only) or daily oral vitamin D3 (800 IU) calcium (1 g) | 60 | Fractures | MI or IHD Stroke Death MI or IHD Stroke Death | 78/2649 118/2649 438/2649 44/1306 60/1306 221/1306 | 84/2643 104/2643 460/2643 39/1332 48/1332 217/1332 |
| Wactawski- wende, ¹⁰⁴ 2006 | 62 | 3628 2 | Older women living in the community (US) | Daily oral vitamin D3 (400 IU)calcium (1 g) | 84 | Fractures and colorectal cancer | MI or IHD Stroke Death | 411/18176 362/18176 744/18176 | 390/18106 377/18106 807/18106 |

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| | | | | | | | | | |
|-----------------------------------|----|------|--|---|----|------------------------|--------------------|--------------------|--------------------|
| Smith, ²²⁸ 2007 | 79 | 9440 | Older people | IM vitamin D3 300 000 IU every year | 36 | Fractures | Death | 355/4727 | 354/4713 |
| Lyons, ²²⁹ 2007 | 84 | 3440 | 2,624 women and 816 men living in care homes (Wales, UK) | Four-monthly oral 100,000 IU vitamin D2 | 36 | Fractures | Death | 947/1725 | 953/1715 |
| Sanders, ¹⁰⁰ 2010 | 76 | 2256 | Community- dwelling women≥ 70 years with a high risk of fracture | Yearly dose of 500,000 IU of vitamin D3 | 60 | Falls and fractures | MI or IHD Death | 17/1131 40/1131 | 13/1125 47/1125 |
| Salovaara, ²³⁰ 2010 | 67 | 3432 | Older women | vitamin D3 800 IU + calcium (1g) | 36 | Fracture | Death | 15/1718 | 13/1714 |

*Only trials with more than 1000 participants and 1 year follow-up were included.

Table 2-4: On-going or planned trials of vitamin D evaluating CVD outcomes or risk factors (updated in August 2013)

| Trial | Participants | Dose | Duration /End of study | Outcomes |
|---|--|--|---------------------------|--------------------------------|
| VITamin D and OmegA-3 TriAL (VITAL),2010 | 20000 men (≥60 years) and women (≥65 years) | 2000 IU daily | 2016/2017 | Cancer, CVD |
| Vitamin D Assessment (ViDA), TBA | 5100 men and women aged between 54-80 years of age | 100,000 IU at baseline and then monthly | 2017 | CVD, infections, fractures |
| The Finnish Vitamin D (FIND), 2012 | 18000 Men≥60 and women ≥ 65 years of age | 1600 IU or 3200 IU daily | 2019 | CVD and cancer |
| Vitamin D and Longevity trial (VIDAL), 2012 | 800 people aged between 65-84 years | 100,000 IU vitamin D3 monthly or placebo | 2 years | CVD , cancer, deaths |
| Prevention of Type 2 Diabetes With Vitamin D Supplementation in Subjects With Reduced Glucose Tolerance Detected in the Tromso Study, 2007 | 517 participants with impaired glucose tolerance identified from the Tromso study | 20000 IU every week | 5 years | Development of type 2 diabetes |

Table 2-5: Summary of Mendelian randomisation studies of vitamin D and CVD risk factors and CVD outcomes (updated August 2014)

| Study Author | Number of participants | Dataset | Vitamin D alleles | Outcomes |
|------------------------------|------------------------|--|---|--|
| Ye ²³¹ | 46 368 | Data from populations of European descent. Ely16 and EPIC-Norfolk studies; EPIC-InterAct, the DIAGRAM consortium, ADDITION-Ely, Norfolk Diabetes, and Cambridgeshire); Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) | DHCR7 (related to vitamin D synthesis), CYP2R1 (hepatic 25-hydroxylation), DBP (known as GC; transport), and CYP24A1 (catabolism) | Low 25(OH)D levels may not be causal of type 2 diabetes. |
| Vimalleswaran ²³² | 108 173 | D-CarDia collaboration, the International Consortium on Blood Pressure (ICBP), the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, and the Global Blood Pressure Genetics (Global BPGen) consortium. (individuals from 35 studies) | CYP2R1 and DHCR7 | Increased 25(OH)D might reduce the risk of hypertension. |
| Afzal ²³³ | 96 423 | White Danes aged 20-100. 5037 of these participants had type 2 diabetes. | DHCR7 (related to endogenous production) and CYP2R1 (related to liver conversion) | Genetic variants associated with low 25(OH)D are associated with type 2 diabetes. Low 25(OH)D mediates may explain link between obesity and increased |

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| | | | | |
|------------------------------|--------|--|---|--|
| | | | | risk of diabetes. |
| Strawbridge ²³⁴ | 3,418 | Carotid Intima-Media Thickness and IMT-Progression as Predictors of Vascular Events in a High-Risk European Population (IMPROVE) cohort study. | SNPs in the genes encoding vitamin D binding protein (GC; rs2282679 and rs7041) and 7-dehydrocholesterol reductase/NAD synthetase-1 (DHCR7; rs12785878 and rs3829251) were negatively associated with 25(OH)D levels. | SNPs in GC and DHCR7 were associated with 25(OH)D. |
| Husemoen ²³⁵ | 9061 | Danish population: Inter99 study (6405 adults, 30-60 years) conducted in 1999-2001, MONICA10 study (2656 adults, 41-71 years) conducted in 1993-1994. | Using variations in the vitamin D-binding protein gene and the filaggrin gene as instrumental variables | Possible causal association between 25(OH)D and adiponectin. |
| Skaaby ²³⁶ | 12911 | Monica10 (2,656 individuals aged 40-71 years), Inter99 (6,784 individuals aged 30-60 years), and Health2006 (3,471 individuals aged 18-69 years) conducted in 1993-94, 1999-2001, and 2006-2008, respectively. | filaggrin gene mutations in European descendants R501X and 2282del4. R2447X mutation. | Higher vitamin D status favours a better lipid profile. |
| Vimalleswaran ²³² | 42,024 | Genetic Investigation of Anthropometric Traits (GIANT) consortium. | four vitamin D-related SNPs (DHCR7- rs12785878, CYP2R1- rs10741657, GC- rs2282679, and CYP24A1- rs6013897 | Higher BMI leads to lower 25(OH)D. |

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| | | | | |
|------------------------|------|--|--|---|
| Trummer ²³⁷ | 3316 | Participants scheduled for coronary angiography between 1997 and 2000. | Genotypes of SNPs in the group-specific component gene (GC, rs2282679), 7-dehydrocholesterol reductase gene (DHCR7, rs12785878), and cytochrome P450 IIR-1 gene (CYP2R1, rs10741657) | Genetic variants associated with 25(OH)D did not predict mortality. |
|------------------------|------|--|--|---|

Chapter 3 : Vitamin D and cause-specific mortality in the Whitehall resurvey

Introduction

This chapter presents the findings from the Whitehall resurvey study, where associations of baseline plasma 25(OH)D concentrations with cause-specific mortality were evaluated (published paper included in **Appendix 1**).

Rationale and Aims

In the previous chapter, low concentrations of 25-hydroxy-vitamin D (25[OH]D) were found to be associated with higher risks of cardiovascular risk factors, CVD and all-cause mortality. However, a number of questions remain unanswered. The reported associations of 25(OH)D with CVD could be due to 'reverse causation' where, those who suffer from disease or chronic ill-health tend to remain indoors more often than others and hence have low vitamin D. Alternatively, these associations may reflect residual confounding from known cardiovascular risk factors. Earlier prospective studies were less able to use consistent methodology for seasonal adjustment of blood 25(OH)D levels,^{130, 133, 238} evaluate the effects of reverse causation;^{203, 239} use appropriate and comparable regression models,²⁰⁶ and select participants more representative of the healthy population (references are representative) minimising the chances of selecting those with pre-existing disease.^{197, 201} The study population size and the follow-up duration were limiting factors in evaluating the strengths and associations with cause-specific mortality.

The plasma 25(OH)D levels that confer the lowest risks for CVD outcomes are unclear and whether possible harm could occur at higher concentrations of 25(OH)D is unknown. The relevance of measuring 25(OH)D concentrations in the general population, especially for non-skeletal outcomes is not known.

In the present study, we aimed to evaluate the associations of baseline 25(OH)D and cause-specific mortality, among surviving participants of the original Whitehall Study who completed a resurvey in 1997 (detailed below).²⁴⁰ The aims of this analysis were to:

- (i) examine prospective associations of 25(OH)D with cardiovascular risk factors;
- (ii) establish the shape, strength and consistency of associations with CVD mortality, and cause-specific mortality;
- (iii) To evaluate the effects of reverse causation and residual confounding; and
- (iv) To establish the concentration of 25(OH)D associated with the lowest risks for CVD mortality.

Methods

Whitehall study and the Whitehall resurvey

The Whitehall study was a prospective study of 19,019 male civil servants who were working in Whitehall, London and recruited between September 1967 and January 1970.²⁴¹ The study involved completing a questionnaire, a medical examination, and collecting blood samples. The pilot Whitehall re-survey was conducted by Robert Clarke from CTSU, University of Oxford, and involved 187 men selected from

amongst the survivors of the original Whitehall study, who were asked to send blood samples by post. Following this successful pilot in 1995, the resurvey was extended to include all surviving 8448 participants during 1997–98.²⁴² The 7044 (83%) respondents to this resurvey were asked to attend their local surgery to have blood and urine samples collected. Complete data on questionnaires and blood results were available on 5409 (77% of respondents) men for the present study.

Data collection and characterisation

The mailed questionnaires asked details of previous medical history (diagnoses of heart attack, angina, stroke, cancer and diabetes), self-reported health status, medications taken in the past month, lifestyle characteristics (e.g. smoking status and alcohol consumption), and last known civil service employment grade. All 7044 (83%) respondents were sent a blood collection kit and asked to attend their local surgery to have a blood sample, and for measurements of blood pressure, height, and weight. The characteristics of the non-responders to the survey are summarised in **Table 3-1** (adapted from Clarke et al).²⁴² The non-responders were older, had a lower previous employment grade, and a higher systolic blood pressure.

Cause-specific mortality follow-up

Information on the cause of death was provided by the Office for National Statistics (England), and included the date and cause of death, until end of August 2010.

Deaths were classified using the International Classification of Disease [ICD] codes (also the commonest type of outcome recording in other large prospective studies).

Blood collection and storage

Whole blood samples (about 10 ml) were returned by post in sealed tubes (Vacutainer; Becton Dickson, Franklin Lakes, N) containing potassium EDTA with 0.34mmol/L of aprotinin. These samples were mailed at room temperature to the CTSU Wolfson laboratory in Oxford, with 96% arriving within 48 hours of blood collection (mean time in post: 1.3 days [range 0-7 days]). The blood was centrifuged, and plasma aliquoted and stored at -40°C . In addition, repeat plasma 25(OH)D concentrations were available on 187 men who were participants of the pilot study. No data were collected on the precise time of day at which the blood samples were collected.

The IDS-iSYS 25 (OH) D assay

Plasma 25(OH)D concentrations were measured using an automated immunoassay on the IDS-iSYS analyser (Immunodiagnostic systems, Boldon, England). Circulating 25(OH)D is stable in stored plasma samples.⁵⁷ The IDS-iSYS 25 (OH) D assay standardisation is traceable to ultraviolet quantification and calibration was verified by comparison with an ID-LC-MS/MS method.

Assay validation and performances were assessed at the CTSU Wolfson laboratory. The IDS-iSYS 25(OH)D assay was comparable for accuracy when using the NIST samples (National Institute of Standards and Technology samples) as reference. Testing the DEQAS samples (Vitamin D External Quality Assessment Scheme samples)²⁴³ indicated the IDS-iSYS assay was systematically overestimating

concentrations compared with the mean obtained for all 25(OH)D assays (Figure in **Appendix 2**). Hence, a linear correction, multiplying all values by 0.757, was applied to measured 25(OH)D concentrations. Precision checking was satisfactory and a brief summary of the methods used in the assay development by our Biochemistry colleagues is included in **Appendix 2**.

Biomarkers associated with CVD risk

Plasma concentrations of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A₁ (Apo A₁) and apolipoprotein B (Apo B) as well as biomarkers of inflammation (C-reactive protein [CRP], fibrinogen and albumin) and cystatin C were measured.²⁴⁴ Estimated glomerular filtration rate (eGFR, in mL min/1.73 m²) was calculated from cystatin C concentration using the formula $eGFR = 80.35/\text{cystatin C [in mg/L]}^{-4.32}$.²⁴⁵

Statistical methods

Plasma 25(OH)D concentrations were analysed on the natural logarithmic scale to normalise the distribution. Since plasma 25(OH)D varied throughout the year with season, values were adjusted for month of blood sampling. This was done by adding to each participant's log 25(OH)D measurement, the difference between the overall mean log 25(OH)D concentration (seen in all men) and the mean observed just among men sampled in the same month (calculated from the sample collection date). Since the majority of men were examined in the summer of 1997, when 25(OH)D concentrations was highest, this adjustment tended to shift values towards those seen in summer rather than winter months. Participants were then

classified into five equal groups based on the season-adjusted 25(OH)D concentration. The means and prevalence of other baseline characteristics, adjusted for age, were estimated (with appropriate tests for linear trend between log 25(OH)D and the risk factor performed using, respectively, linear or logistic regression adjusted for age).

To further assess the shape of the association between 25(OH)D and mortality, men in the top and bottom fifths were divided in half to better characterise the dose-response relationships at the extreme concentrations. Relative risks (estimated by hazard ratios in Cox models) were estimated for each group relative to the lowest group and shown as “floating absolute risks” (merely ascribes a 95% confidence interval [CI] to the RR in every group).²⁴⁶ The average relative risk corresponding to a doubling in 25(OH)D concentration (approximately a 2 standard deviation increase on the log scale) was also estimated. The proportionality assumption of the Cox model was assessed using the method described by Grambsch and Therneau.²⁴⁷ Analyses were done before and after adjustment for age, prior history of disease (myocardial infarction, angina, stroke, cancer or diabetes), self-reported health status (on a four point scale), ability to perform particular activities of daily living (based on a 15-point questionnaire), smoking status (current smoker, ex-smoker, never smoker), alcohol consumption, last known employment grade, blood pressure (both at entry to Whitehall study in 1967-70 and at resurvey in 1997-98, as well as treatment for hypertension at resurvey), body mass index, blood lipids and apolipoproteins (LDL-C, HDL-C, Apo A₁, Apo B), markers of inflammation (C-reactive protein, albumin, fibrinogen), and eGFR.

To assess the effects of reverse causality, analyses were repeated separately in men with and without a prior history of disease (defined as above), while further analyses excluded deaths during the first five years of follow-up. All p-values were two sided and p-values <0.05 were deemed conventionally significant. Analyses were done using SAS version 9.1 (SAS Institute, Cary, NC, USA) and R version 2.11.1 (www.r-project.org).

Results

Key results are presented here (published paper in **Appendix 1**).⁹ The Whitehall resurvey participants included 5409 men and their baseline characteristics are summarised in **Table 3-2**. The mean age of participants at resurvey was 76.9 (SD 4.9) years, and about one-third (1841 men) had a history of prior vascular disease, cancer or diabetes. The majority (87%) were non-smokers, while 78% were self-reported 'current' alcohol drinkers.

Distribution of 25(OH)D concentrations at baseline

Plasma concentrations of 25(OH)D varied by month of blood collection. Absolute values for plasma 25(OH)D concentrations varied widely during the year with peak levels being seen at the end of summer. Although the mean plasma 25(OH)D were relatively higher than expected in November and December. However, the number of samples collected was fewer and hence mean values may be skewed by extreme values from participants who may have had more exposure to sun in the preceding month. The mean concentrations after adjustment for month of blood collection

show a log-normal distribution (**Figure 3-1**). Median 25(OH)D concentration (standardised for month of blood collection) was 56 nmol/L (interquartile range 45-67 nmol/L). Repeated measurements were available in 187 men, taken 1.5 years apart, and the self-correlation in log 25(OH)D was 0.64.

At any given age, men with lower 25(OH)D concentrations were likely to have a history of vascular disease, cancer or diabetes, and diagnosed with hypertension or taking treatment for hypertension, than men with higher concentrations. Measured systolic blood pressure at resurvey in 1997 was only weakly related with 25(OH)D concentrations, and the slight increase in blood pressure across the quintiles may be a play of chance. The blood pressure at the initial examination for the Whitehall study in 1967-70 was unrelated with 25(OH)D concentrations. Men with higher 25(OH)D had lower mean BMI, were less likely to have been of manual/clerical grade at retirement, had higher mean LDL-C, HDL-C, ApoA₁ and albumin concentrations, and lower C-reactive protein and fibrinogen concentrations, than men with lower 25(OH)D concentrations.

Association of 25(OH)D with vascular and non-vascular mortality

Among the 5409 participants, 3215 men died over 50,000 person years of follow-up (death rate: 6.4% per year; mean follow-up among survivors 13 years), including 1358 deaths (2.7% per year) from vascular causes and 1857 deaths (3.7% per year) from non-vascular causes. Among the 3568 men without a history of vascular

disease, cancer or diabetes, there were 727 deaths (2.0% per year) from vascular causes and 1124 deaths (3.1% per year) from non-vascular causes.

After classifying men into seven groups based on season-adjusted 25(OH)D concentration, higher concentrations of 25(OH)D were inversely and, on the log-log scale, approximately linearly related to the risk of vascular mortality, at least throughout the range 40 to 90 nmol/L, of non-vascular mortality in age-adjusted models (**Figure 3-2**). The shape of these associations were broadly similar for both vascular and non-vascular mortality (with some attenuation of risk for non-vascular mortality with 25(OH)D > 80 nmol/L), and, for both outcomes, associations were consistent among men with and without a prior history of vascular disease, cancer or diabetes.

Effect of adjustment for potential confounders

Given age, a doubling in 25(OH)D concentration (corresponding to a $\ln(2)$ absolute difference – about 2 standard deviations – on the log-scale) was, on average, associated with a 34% lower risk of vascular mortality (RR 0.66, 95% CI 0.58-0.75) and a 36% lower risk of non-vascular mortality (RR 0.64, 95% CI 0.58-0.72) (**Figure 3-3**). After adjustment for prior disease (including self-reported measures of health and frailty), established vascular risk factors, markers of inflammation and renal function, this was reduced to a 20% lower risk of vascular mortality (RR 0.80; 95% CI 0.70-0.91) and a 23% lower risk of non-vascular mortality (RR 0.77; 95%CI 0.69-0.86). The substantial change in the χ^2 statistic associated with 25 (OH) D

concentrations with these adjustments (from 41.1 to 11.5 for vascular death and 63.3 to 21.4 for non-vascular death) suggest that a large part of these associations was due to confounding, principally by prior disease. There was no evidence that the relative risks associated with a doubling in baseline 25(OH)D concentration varied during follow-up (p-values for test of proportionality assumption: $p=0.48$ for vascular mortality and $p=0.13$ for non-vascular mortality). Associations with particular types of vascular and non-vascular death (e.g. IHD, stroke, cancer and respiratory death), after adjustment for measured confounders, were broadly similar to the overall relative risks seen for vascular and non-vascular mortality (**Figure 3-4**). The findings for participants with no prior disease at resurvey were similar to those of the overall study population (**Figures 3-5 and 3-6**). Results were also broadly similar after exclusion of deaths within the first five years of follow-up (to further reduce the possible effect of reverse causality) (**Figure 3-7**).

Conclusions

In this prospective study of older men, baseline concentrations of plasma 25(OH)D were found to be predictive of the risks of both all-cause and cardiovascular mortality. In the next chapter, we included the findings of the Whitehall Study with data from other prospective studies reporting associations of vitamin D with cardiovascular or all-cause mortality.

Chapter 3: Figures and Tables

Table 3-1: Characteristics of non-responders in the Whitehall resurvey*

| | Total number of participants | Non-responders to long questionnaires (%) | Blood samples not sent (%) |
|--------------------------|------------------------------|---|----------------------------|
| Age at re-survey | | | |
| <75 | 3027 | 24 | 19 |
| 75-79 | | 24 | 20 |
| 80+ | 2940 | 37 | 33 |
| | 2570 | | |
| Estimated income in 1997 | | | |
| £25300+ | 2611 | 23 | 20 |
| £18800 – 25299 | 2610 | 27 | 23 |
| <18800 | 2613 | 32 | 26 |
| Employment grade | | | |
| Administrative | 555 | 20 | 14 |
| Executive/Prof/Dip | 6743 | 25 | 21 |
| Clerical / other | 1239 | 46 | 38 |
| Residence | | | |
| London | 2591 | 33 | 27 |
| Outside London | 5946 | 26 | 21 |
| Smoking status | | | |
| Current/ex-smoker | 6455 | 29 | 24 |
| Never | 2078 | 24 | 20 |
| Systolic blood pressure | | | |
| Group I | 2170 | 25 | 20 |
| Group V | 1125 | 33 | 28 |
| All | 8537 | 18 | 20 |

*Among the non-responders, 9% actively refused participation, 2% had died by the time, 2% moved address and no information was available in 5%.

Table 3-2: Baseline characteristics of participants in the Whitehall resurvey

| Baseline characteristic | Fifth of baseline 25(OH)D * | | | | | | | | p [†] |
|--|-----------------------------|------------------|---------------|--------------|--------------|--------------|--------------|--------------|----------------|
| | All men | No prior disease | Prior disease | I | II | III | IV | V | |
| Number of men | 5409 | 3568 | 1841 | 1081 | 1082 | 1082 | 1082 | 1082 | ... |
| Age, years | 76.9 (4.9) | 76.5 (4.8) | 77.6 (5.0) | 78.9 (5.2) | 77.1 (5.1) | 76.7 (4.6) | 76.1 (4.5) | 75.5 (4.3) | ... |
| 25(OH)D, nmol/L | 56 (45-67) | 57 (47-68) | 54 (43-65) | 36 (5) | 48 (3) | 56 (2) | 65 (3) | 83 (18) | ... |
| Medical history, % | | | | | | | | | |
| IHD | 19.9 | 0.0 | 58.4 | 22.0 | 20.5 | 20.3 | 19.8 | 16.5 | 0.002 |
| Stroke | 7.2 | 0.0 | 21.1 | 9.3 | 6.6 | 6.7 | 6.7 | 5.9 | 0.001 |
| CVD | 24.9 | 0.0 | 73.3 | 28.1 | 25.4 | 25.4 | 24.6 | 20.7 | <0.001 |
| Diabetes | 5.9 | 0.0 | 17.4 | 8.8 | 6.7 | 5.8 | 2.8 | 4.0 | <0.001 |
| Cancer (not skin) | 7.9 | 0.0 | 23.2 | 10.1 | 8.1 | 7.4 | 6.8 | 6.5 | 0.006 |
| Self-reported health good/excellent | 77.4 | 85.3 | 62.0 | 67.1 | 75.8 | 79.9 | 80.5 | 85.5 | <0.001 |
| Manual/clerical socio-economic grade at baseline | 17.4 | 17.7 | 16.8 | 20.2 | 19.4 | 16.2 | 15.8 | 14.9 | <0.001 |
| Lifestyle, % | | | | | | | | | |
| Current tobacco smoker | 12.7 | 14.1 | 10.2 | 16.8 | 12.3 | 12.7 | 10.1 | 11.4 | <0.001 |
| Current alcohol drinker | 77.9 | 79.2 | 75.4 | 73.3 | 75.8 | 80.5 | 79.5 | 80.8 | <0.001 |
| Blood pressure and body mass | | | | | | | | | |
| Diagnosis of hypertension / use of blood pressure lowering drugs | 42.0 | 33.1 | 59.3 | 45.0 | 43.1 | 41.4 | 40.2 | 40.5 | 0.003 |
| Systolic blood pressure, mm Hg | 144.8 (20.1) | 145.4 (19.8) | 143.7 (20.7) | 144.0 (20.5) | 144.6 (20.1) | 145.2 (20.1) | 144.9 (20.2) | 145.5 (20.3) | 0.05 |
| Diastolic blood pressure, mm Hg | 80.2 (10.8) | 80.9 (10.7) | 78.8 (10.9) | 79.6 (11.0) | 80.4 (10.8) | 80.1 (10.8) | 80.1 (10.8) | 80.5 (10.8) | 0.10 |
| Body mass index, kg/m ² | 25.2 (3.2) | 25.1 (3.2) | 25.4 (3.3) | 25.5 (3.3) | 25.4 (3.2) | 25.4 (3.2) | 25.1 (3.2) | 24.8 (3.2) | <0.001 |

Whitehall resurvey

| Baseline characteristic | Fifth of baseline 25(OH)D * | | | | | | | | p† |
|---|-----------------------------|------------------|---------------|--------------|--------------|--------------|--------------|--------------|--------|
| | All men | No prior disease | Prior disease | I | II | III | IV | V | |
| Blood pressure measured in 1967-1970 (~30 years earlier), mm Hg | | | | | | | | | |
| Systolic blood pressure | 130.8 (17.6) | 129.5 (17.2) | 133.3 (18.2) | 130.5 (17.9) | 130.9 (17.5) | 131.3 (17.5) | 130.5 (17.6) | 130.5 (17.7) | 0.57 |
| Diastolic blood pressure | 80.3 (12.1) | 79.4 (11.7) | 82.0 (12.6) | 80.1 (12.3) | 80.6 (12.1) | 80.8 (12.1) | 80.1 (12.1) | 79.9 (12.2) | 0.35 |
| Laboratory measurements | | | | | | | | | |
| LDL-C, mmol/L | 3.37 (0.79) | 3.40 (0.78) | 3.31 (0.80) | 3.28 (0.79) | 3.36 (0.78) | 3.42 (0.78) | 3.42 (0.78) | 3.36 (0.78) | <0.001 |
| HDL-C, mmol/L | 1.09 (0.38) | 1.12 (0.37) | 1.04 (0.38) | 1.06 (0.38) | 1.06 (0.38) | 1.08 (0.38) | 1.11 (0.38) | 1.17 (0.38) | <0.001 |
| Apolipoprotein A ₁ , g/L | 0.95 (0.15) | 0.96 (0.14) | 0.93 (0.15) | 0.93 (0.15) | 0.94 (0.14) | 0.95 (0.14) | 0.96 (0.14) | 0.98 (0.15) | <0.001 |
| Apolipoprotein B, g/L | 0.87 (0.23) | 0.87 (0.23) | 0.87 (0.24) | 0.85 (0.23) | 0.87 (0.23) | 0.88 (0.23) | 0.88 (0.23) | 0.85 (0.23) | 0.38 |
| C-reactive protein, mg/L | 3.7 (7.7) | 3.4 (7.2) | 4.4 (8.5) | 4.3 (7.8) | 3.7 (7.7) | 3.6 (7.7) | 3.5 (7.7) | 3.5 (7.7) | <0.001 |
| Albumin, g/L | 39.7 (3.0) | 39.8 (2.8) | 39.3 (3.2) | 39.3 (2.9) | 39.6 (2.9) | 39.7 (2.9) | 39.9 (2.9) | 39.9 (2.9) | <0.001 |
| Fibrinogen, umol/L | 3.5 (0.8) | 3.5 (0.8) | 3.6 (0.9) | 3.6 (0.9) | 3.6 (0.8) | 3.5 (0.8) | 3.5 (0.8) | 3.5 (0.9) | 0.02 |
| Renal function | | | | | | | | | |
| eGFR, mL/min/1.73m ² | 72.2 (15.3) | 74.0 (14.4) | 68.6 (16.4) | 69.8 (14.7) | 71.7 (14.4) | 72.9 (14.4) | 73.0 (14.4) | 73.5 (14.5) | <0.001 |

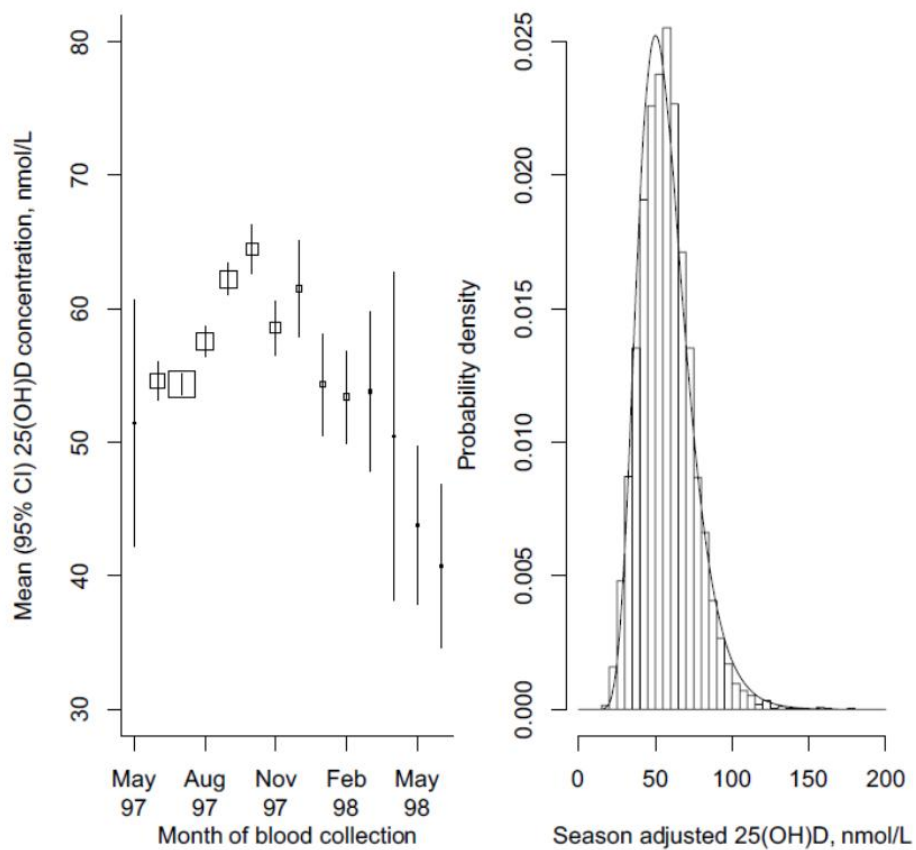
Mean (SD), median (IQR) or n (%) shown.

IHD = Ischaemic heart disease (recall of diagnosis of myocardial infarction or angina)

* With the exception of age and vitamin D, estimates are adjusted for age differences across vitamin D groups

† test of linear trend between log(25[OH]D) concentration and baseline characteristic after adjustment for age

Figure 3-1: Raw and unadjusted mean 25(OH)D concentrations by month of resurvey (left) and distribution of season-adjusted 25(OH)D (right)



In the right panel, the solid curve which nearly superimposes the histogram represents the log-normal distribution with mean and standard deviation taken from the sample 25(OH)D data (on the log scale)

To convert 25(OH)D from nmol/L to ng/mL, divide by 2.496

Figure 3-2: Age-adjusted relevance of measured 25(OH)D for vascular and non-vascular mortality in old age, overall and separately in men with and without prior disease

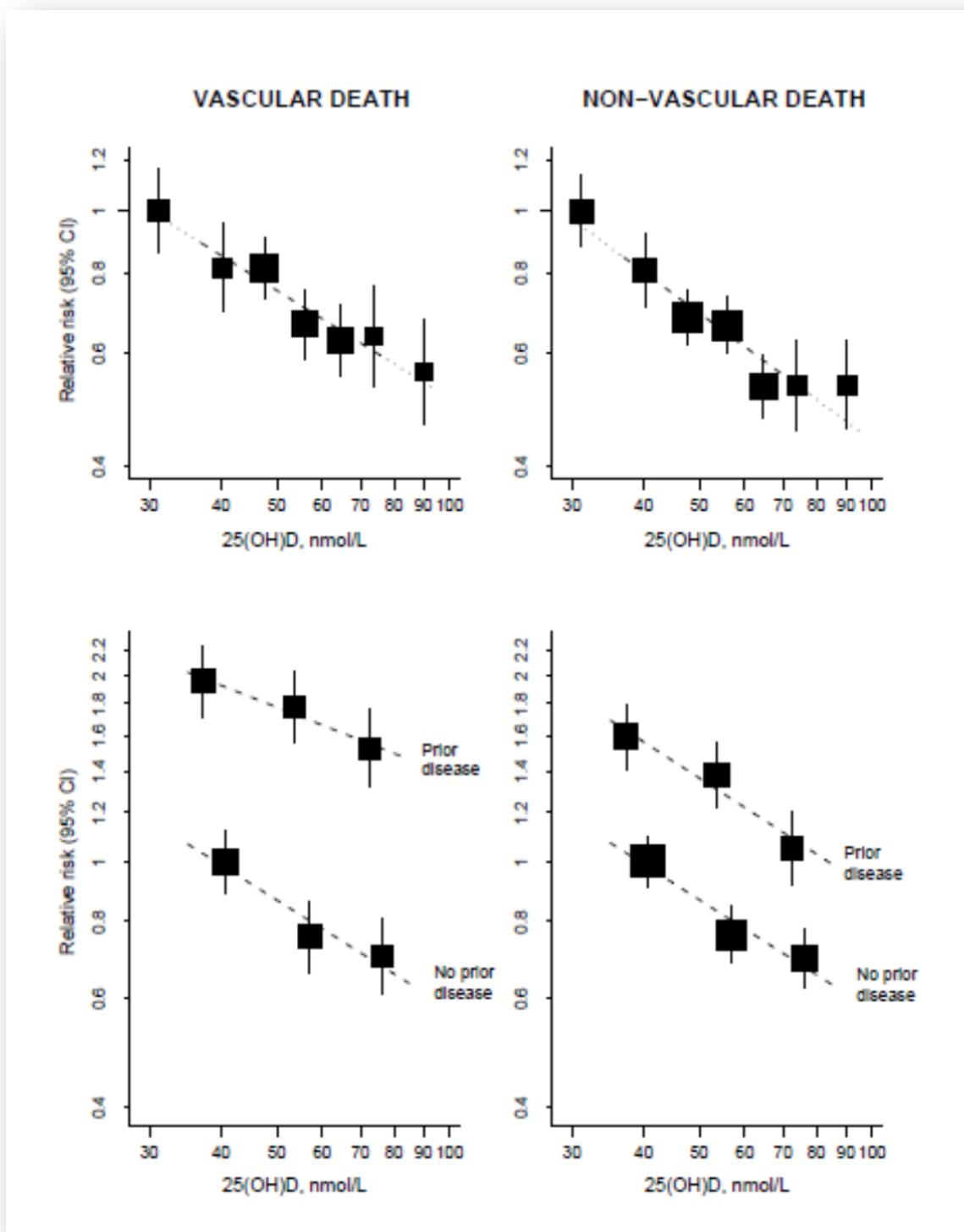


Figure 3-3: Effect of adjustment for known risk factors on the association between measured 25(OH)D and vascular and non-vascular mortality

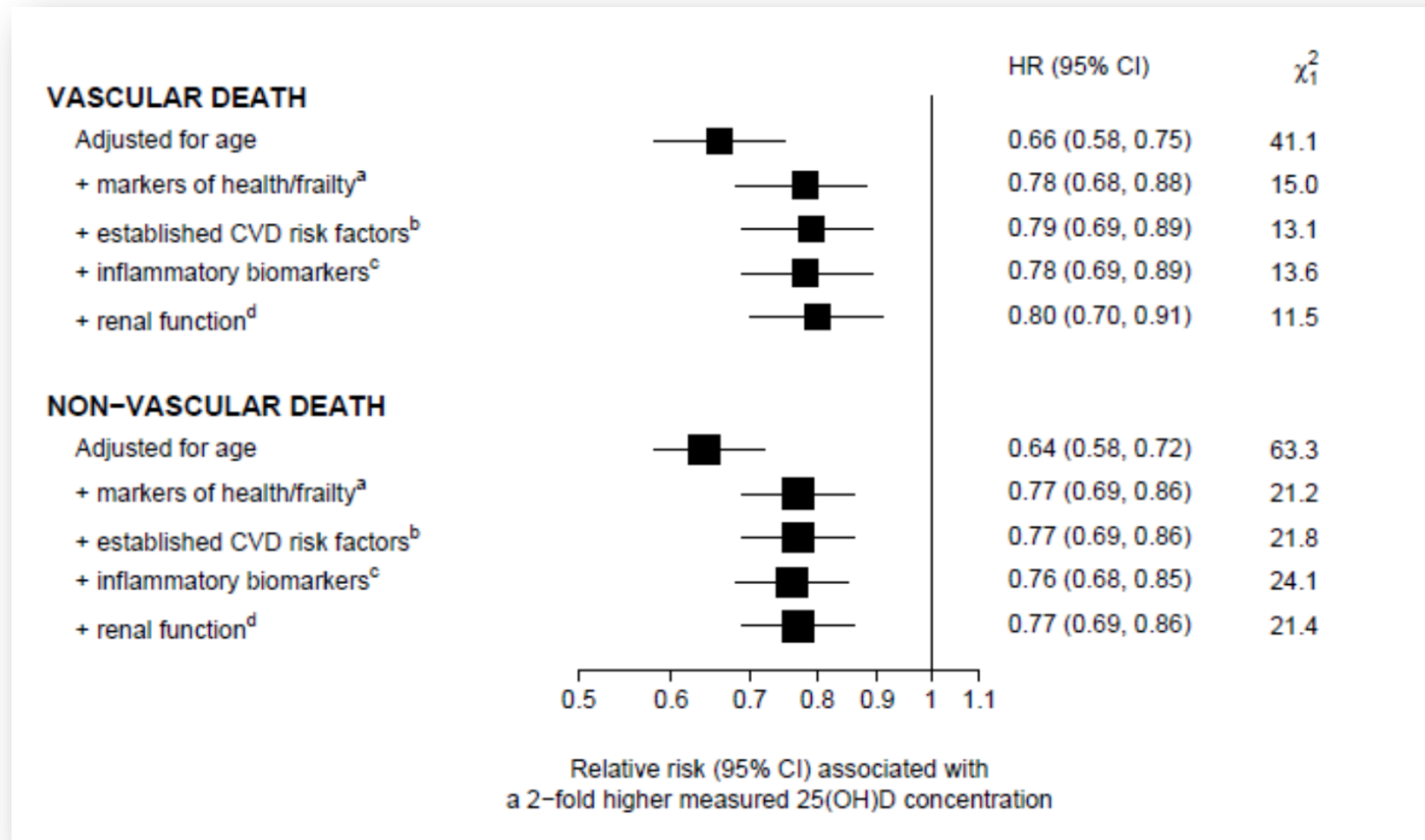


Figure 3-4: Association between measured 25(OH)D and cause-specific mortality after adjustment for measured confounders

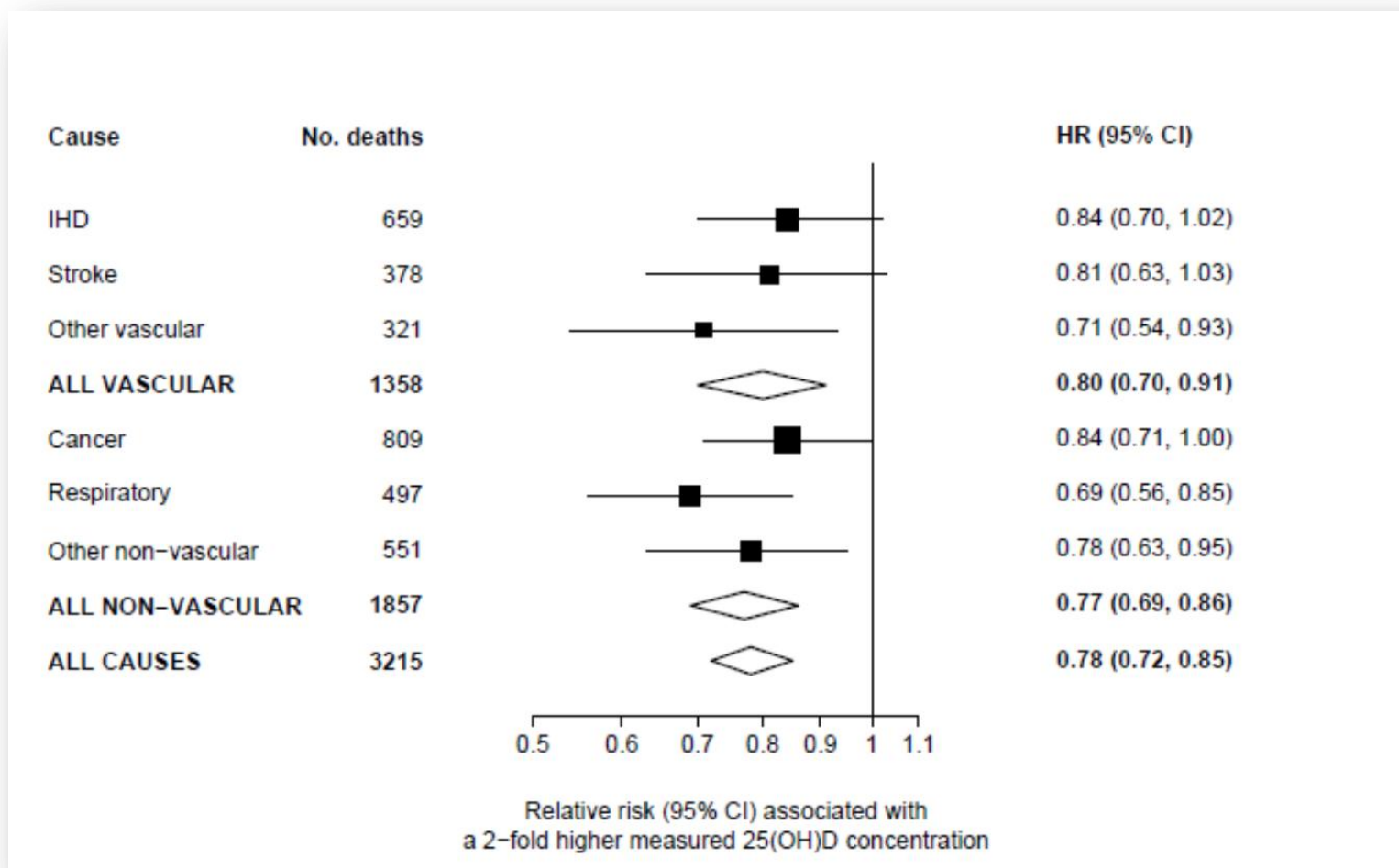
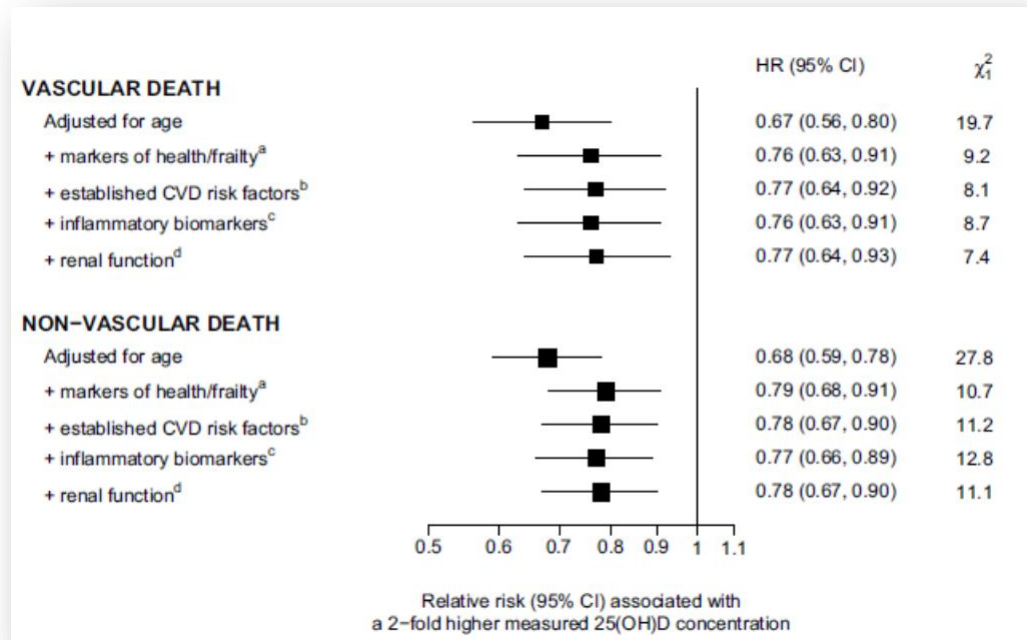


Figure 3-5 Effect of adjustment for known risk factors on the association between measured 25(OH)D and vascular and non-vascular mortality among men without prior disease



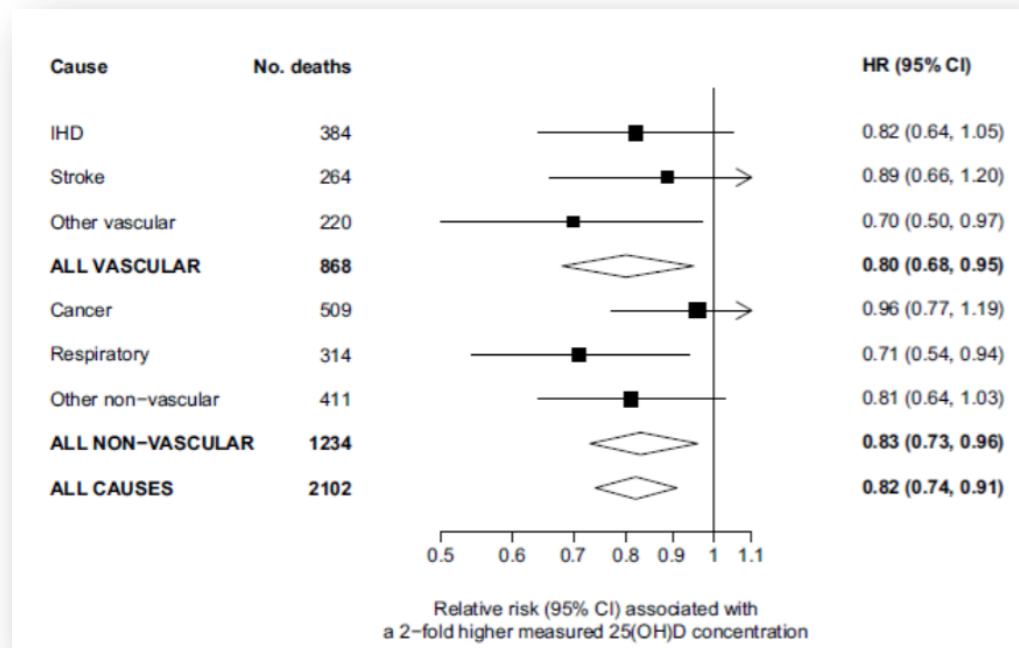
a: self-reported health/frailty only (because this analysis already excludes men with known prior cardiovascular disease, cancer or diabetes)

b: smoking status (current/ex/never); drinking status (current/non); grade of employment; LDL-C, HDL-C, apo A1, apo B, BMI and blood pressure (recall [in 1997] of a diagnosis of hypertension, treatment [in 1997] for hypertension and measured systolic and diastolic blood pressure in both 1997 and in ~1970)

c: albumin, fibrinogen and C-reactive protein

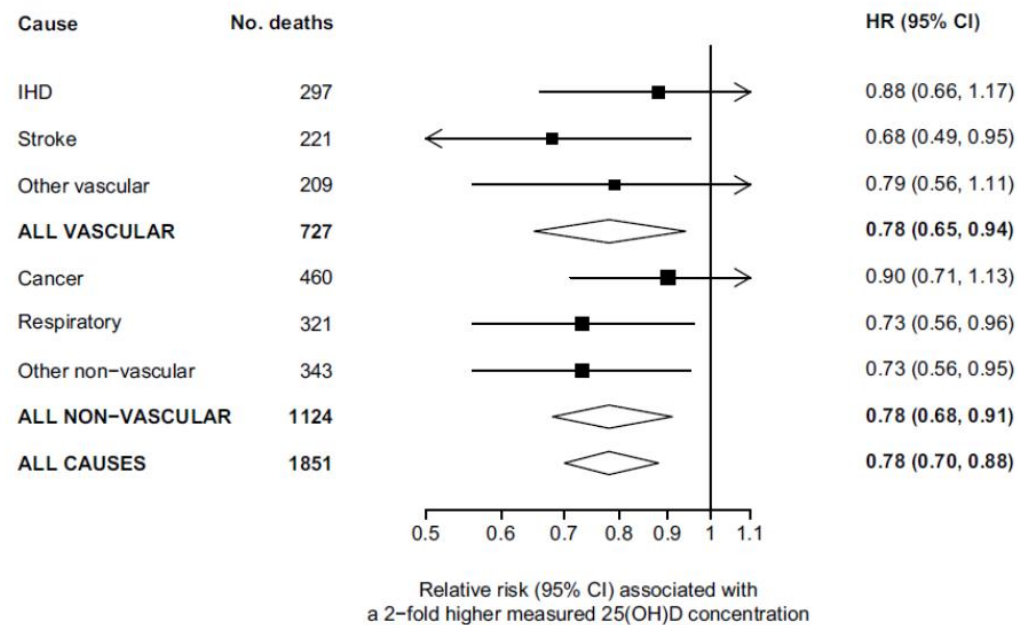
d: eGFR

Figure 3-6: Association between measured 25(OH)D and cause-specific mortality after adjustment for measured confounders among men without prior disease



IHD=ischaemic heart disease. Analyses are adjusted for smoking status (current/ex/never), drinking status (current/non), recall of a diagnosis of IHD, stroke, cancer or diabetes, self-reported health/frailty, employment grade, LDL-C, HDL-C, apo A1, apo B, BMI, markers of inflammation (albumin, fibrinogen and C-reactive protein), recall (in 1997) of a diagnosis of hypertension, treatment (in 1997) for hypertension and measured systolic and diastolic blood pressure in both 1997 and in ~1970, and cystatin C

Figure 3-7: Association between 25(OH)D concentration and cause-specific mortality after adjustment for measured confounders, excluding deaths in the first 5 years



Excludes men with a prior diagnosis of cardiovascular disease, cancer or diabetes; IHD=ischaemic heart disease.

Analyses are adjusted for smoking status (current/ex/never), drinking status (current/non), self-reported health/frailty, employment grade, LDL-C, HDL-C, apo A1, apo B, BMI, markers of inflammation (albumin, fibrinogen and C-reactive protein), recall (in 1997) of a diagnosis of hypertension, treatment (in 1997) for hypertension and measured systolic and diastolic blood pressure in both 1997 and in ~1970, and cystatin C

Chapter 4 : Meta-analyses of prospective studies of vitamin D and the risks of CVD and all-cause mortality

Introduction

This chapter evaluates the consistency of the findings from the prospective Whitehall study presented in Chapter 3 when compared to the results of other prospective studies reporting on CVD or all-cause mortality (**Appendix 1**).

Rationale and Aims

Low concentrations of 25-hydroxy-vitamin D (25[OH]D) at baseline were shown to be inversely associated with higher risks of cardiovascular deaths and all-cause mortality among older men living in the UK in the Whitehall study. These findings are intriguing as the associations lack specificity, in that baseline 25(OH)D concentrations are associated to both CVD and non-CVD causes of mortality. It is also unclear whether these reported associations among older men living in the UK, are similar to those reported in other studies which included people living in other geographical locations, women, or younger participants. If the reported associations between baseline plasma 25(OH)D levels and CVD mortality in the Whitehall study are similar to those reported elsewhere, the findings may be generalisable to the general population and the causality of these associations would need testing in randomised clinical trials. Further, it is unclear whether the shape and size of the associations reported from the Whitehall resurvey are similar in shape and consistency in other prospective studies.

In the present study, we evaluate the comparability and consistency of the associations of baseline 25(OH)D and cause-specific mortality from the Whitehall Resurvey, by including this data in meta-analyses of other prospective studies that reported on associations of baseline 25(OH)D and outcomes of cardiovascular and all-cause mortality.

The main aims of this analysis were to:

- (i) examine the strength of the prospective associations of baseline 25(OH)D with cardiovascular mortality and all-cause mortality;
- (ii) examine the consistency of the relationships between baseline concentrations of plasma 25(OH)D and CVD and all-cause mortality.

Methods

Systematic search methods of prospective studies

Published reports of prospective studies and case-control studies reporting on associations of 25(OH)D with either vascular or all-cause mortality were identified through a systematic electronic search of the three electronic databases (PubMed, Embase and Cochrane). The step-wise search methods used in PubMed is outlined in **Figure 4-1**. This search was complimented by searching the reference lists of the selected articles, and reviewing reference lists of studies included in previous meta-analyses of prospective studies of 25(OH)D. The studies were included if they were in English and published before January 2012. We aimed to include all prospective studies that recruited participants with characteristics that were broadly

comparable with those of the general population, and excluded studies that selected participants predominantly from those with pre-existing conditions (see eligibility below). This was to minimise the effects of reverse causation on the outcomes reported.

We included studies where participants were mainly older people living in the community, as older prospective studies of vitamin D included older participants and were designed to evaluate their bone outcomes; excluding such studies could introduce a bias (significantly reduces the sample size, potentially exclude information if the outcomes were either null or inconsistent). However, we excluded those that were conducted in the very frail and included the extremes of age.

Selection criteria

Inclusion criteria:

Studies were included if:

- (i) It was a prospective (or nested case-control) study;
- (ii) It included more than 200 adult participants, predominantly recruited from the general population;
- (iii) The study reported data on baseline 25(OH)D concentrations and on risks of death from CVD or deaths from all causes.

Exclusion criteria:

Studies were excluded if:

- (i) participant selection was predominantly from among patients with prior disease (cancer, vascular, or renal disease);
- (ii) participants were predominantly recruited from patients with CVD risk factors (diabetes and hypertension) only;
- (iii) they were studies that were conducted among very frail or nursing home residents (they were not considered to be representative of the general population);
- (iv) study included participants who were enrolled in clinical trials of vitamin D;
- (v) such studies were other meta-analyses.

Studies were also excluded if they were reporting from the same population (e.g. the NHANES cohort); the largest study with the most deaths was included in such cases.

Principle outcomes

The key outcomes of interest were the relative-risks of:

- (i) CVD mortality
- (ii) All-cause mortality, based on baseline 25(OH)D concentrations.

Studies included generally defined causes of death by ICD codes from either death certificates or by physician reviews; one large study reported a composite outcome which included CVD death.¹²⁸ As the numbers of studies were limited and included studies that reported other outcomes, we also included an indication of the quality

of the information deduced from such studies by reporting on information about the number of participants studied, the duration of follow-up, the inclusion of the methodology for correction of seasonal variation in 25(OH)D concentrations, and the number and type of confounders used in the adjustment regression models.

Relative risk ratios (RRs) from each report were extracted (commonly hazard ratios from Cox regression models) along with their 95% CI for vascular and all-cause mortality, and independently verified by two researchers (Robert Clarke and Joseph Tomson). For each study, the most fully adjusted RR and its 95% CI were recorded. Regression models that included calcium and phosphate in their adjustment were not included (as these are on the causal pathway; the established action of vitamin D is the regulation of calcium and phosphate metabolism).

Consistent with PRISMA Guidelines, studies included in the meta-analyses were evaluated (**Table 4-2**) for: design (prospective or retrospective), characteristics of participants, representative of the general population (i.e. representativeness based on eligibility criteria), appropriate evaluation of relevant outcomes, duration of follow-up and the appropriate use of multivariate regression analyses. However, a formal scoring system was not used. It was difficult to ascertain the quality of outcomes evaluated in the published literature (including risks of detection and attrition biases). Hence, only studies that either included participants with pre-existing disease (such as diabetes, cancer, kidney disease or CVD) or those with a significant likelihood of underlying chronic disease (e.g. extremes of age or frailty) were excluded.

Statistical analysis

Where necessary, the RRs were recalibrated to correspond to the top vs. bottom quarter of the 25(OH)D distribution (most common approach in the studies included in the analyses).²⁴⁸ This was done by estimating the number of SDs that each published RR would correspond to [on some normal transformation of the underlying 25(OH)D distribution] before recalibrating the log RR (and its standard error) to correspond to a 2.54 SD difference (since 2.54 is the difference in mean values between the top and bottom quarters of a normal distribution). We contacted the principal investigators of selected studies to provide additional data on the SD of 25(OH)D concentration in their cohorts to facilitate standardized comparisons.

Overall summary estimates of the effect were calculated using the Mantel–Haenszel inverse-variance weighted method for meta-analysis. In the forest plots, studies were ordered according to the amount of statistical information they contributed to the overall result [and, for display only, were grouped as being: ‘small’ (providing <1% of the total information provided by all the studies); ‘medium’ (1 to <10%); or ‘large’ (at least 10%)]. Analyses were also carried out to assess the effects of differences in 25(OH)D on mortality after stratifying studies by age, gender and study size using meta-regression. All *p* values were two sided and *p* values < 0.05 were deemed conventionally significant.

Results

Prospective studies included in the meta-analyses

The meta-analyses (which included results from the Whitehall resurvey study) included 12 prospective studies with 4632 vascular deaths and 18 prospective studies with 11,734 deaths from all causes (**Table 4-1**). The information from the Whitehall study was the largest contributor to both the meta-analyses for the risks of mortality with more than 3000 deaths. The prospective study with the longest period of follow-up of 27 years was the Mini-Finland Survey, while the largest number of participants and the shortest follow-up of 1.3 years were in the InterMountain Healthcare study. Although we did not apply an eligibility criteria based on the quality of information provided given the paucity of studies, an indication of the nature and extent of the information provided by these studies are shown in **Table 4-2**.

Relative risks of death between quartiles

Participants with a baseline 25(OH)D concentration in the top quarter of distribution had on average, 21% (95% CI 13-28%) lower vascular mortality (**Figure 4-2**) and 28% (95% CI 24-32%) lower all-cause mortality (**Figure 4-3**) when compared to the lowest quarter. However, the observed relative risks varied inversely with the amount of statistical information provided by each study (i.e. by study size). That is the more extreme estimates of the risks of dying were reported in smaller studies for both vascular and all-cause mortality outcomes.

All-cause mortality by age, gender and baseline 25(OH)D concentrations

When studies were stratified by mean age of the included participants, no differences in risks for all-cause mortality were identified between extreme quartiles (**Table 4-3**). The mean age of the participants in the studies included ranged between 59 to 84 years. When stratifying by gender (**Table 4-4**), three studies included only women and four recruited only men. The relative risks for all-cause mortality in studies recruiting women was 54% lower in women in the top quartile of plasma 25(OH)D than those women in the lowest quartile, while the relative risks were only 25% lower in studies recruiting only men between top versus bottom quartiles ($p=0.05$). However, when after adjusting for age, gender and study size, only study size remained a significant contributor to the heterogeneity in these analyses (**Figure 4-4**). No differences were seen in the relative risks of death, when studies were evaluated by the range of 25(OH)D concentrations between the extreme quartiles, or when stratifying them by published mean concentrations of 25(OH)D for the lowest quarter (published mean 25(OH)D levels for each quartile was available only in 9 prospective studies reporting on all-cause mortality – data not shown).

Conclusion

The findings from the meta-analyses of prospective studies show a consistent shape and trend in the inverse associations between plasma 25(OH)D and the risks of both CVD and all-cause mortality, irrespective of geographic locale, gender or baseline 25(OH)D concentrations. In the next chapter, we discuss the design of a dose-

finding trial of vitamin D, BEST-D, where the trial aims to identify the dose of vitamin D3 supplement to be used in a future trial of vitamin D evaluating cardiovascular and other outcomes.

Chapter 4: Figures and Tables

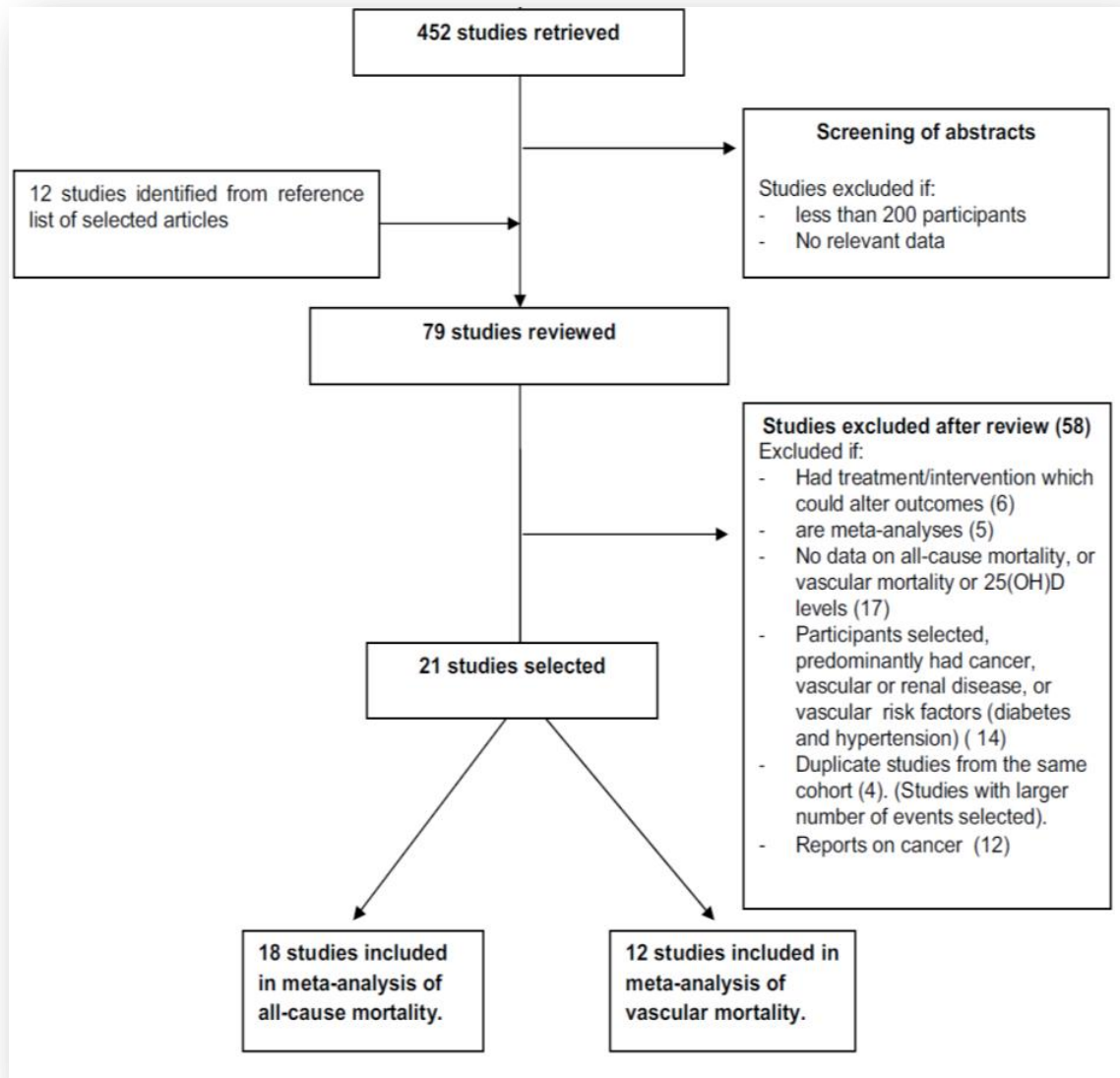
Figure 4-1: Identification of prospective studies of 25(OH)D and mortality

Figure 4-2: Meta-analysis of the relationship between 25(OH)D concentration and vascular mortality

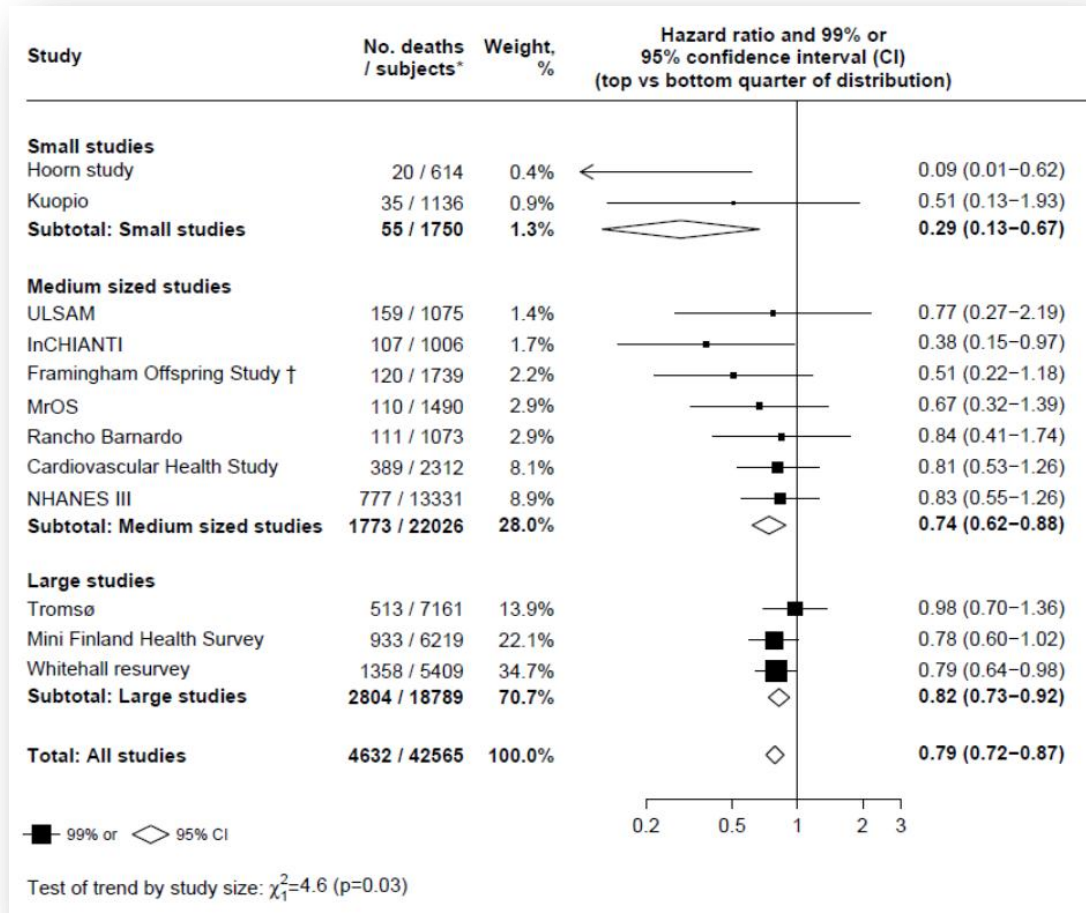


Figure 4-3: Meta-analysis of the relationship between 25(OH)D concentration and all-cause mortality

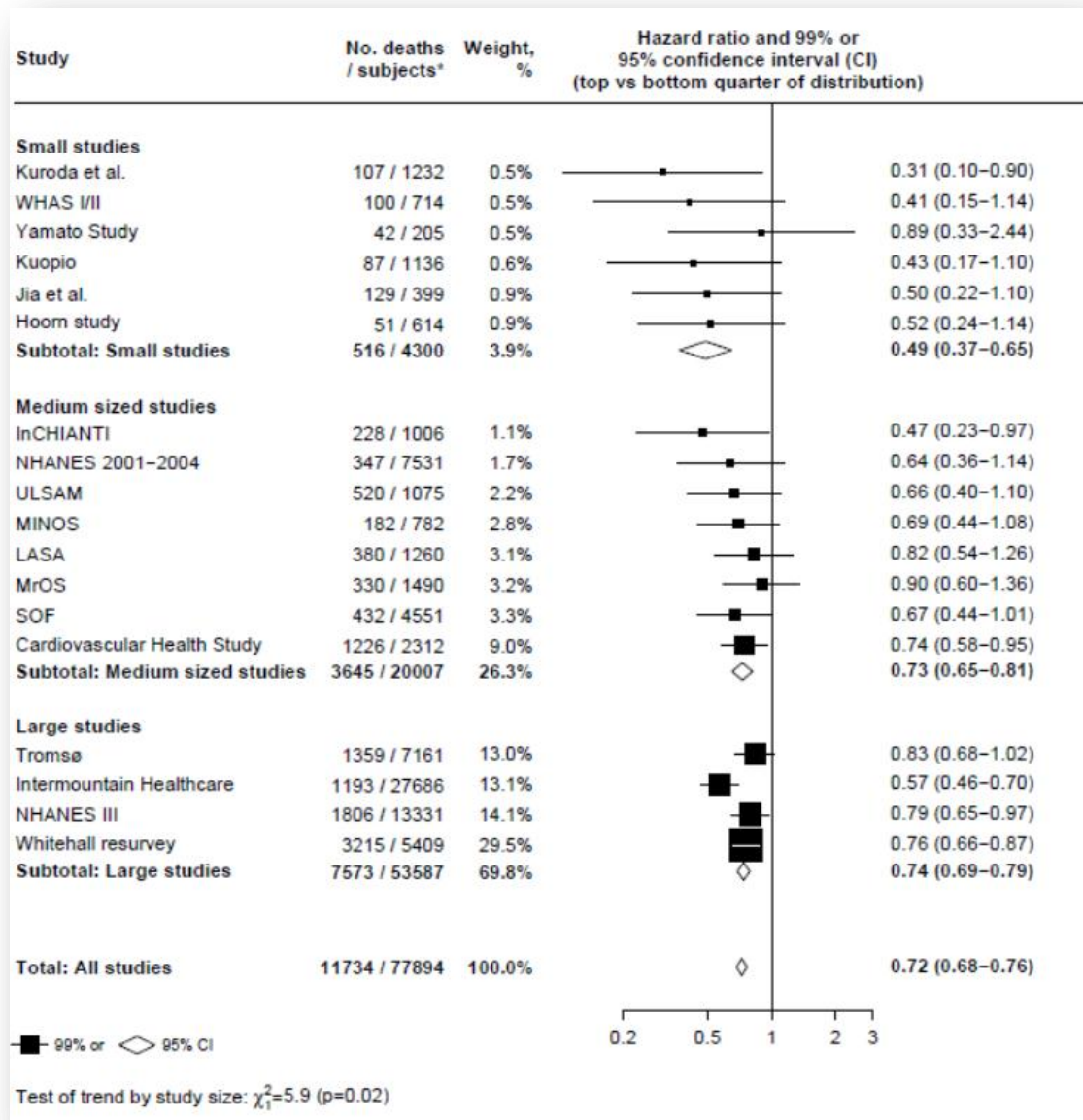
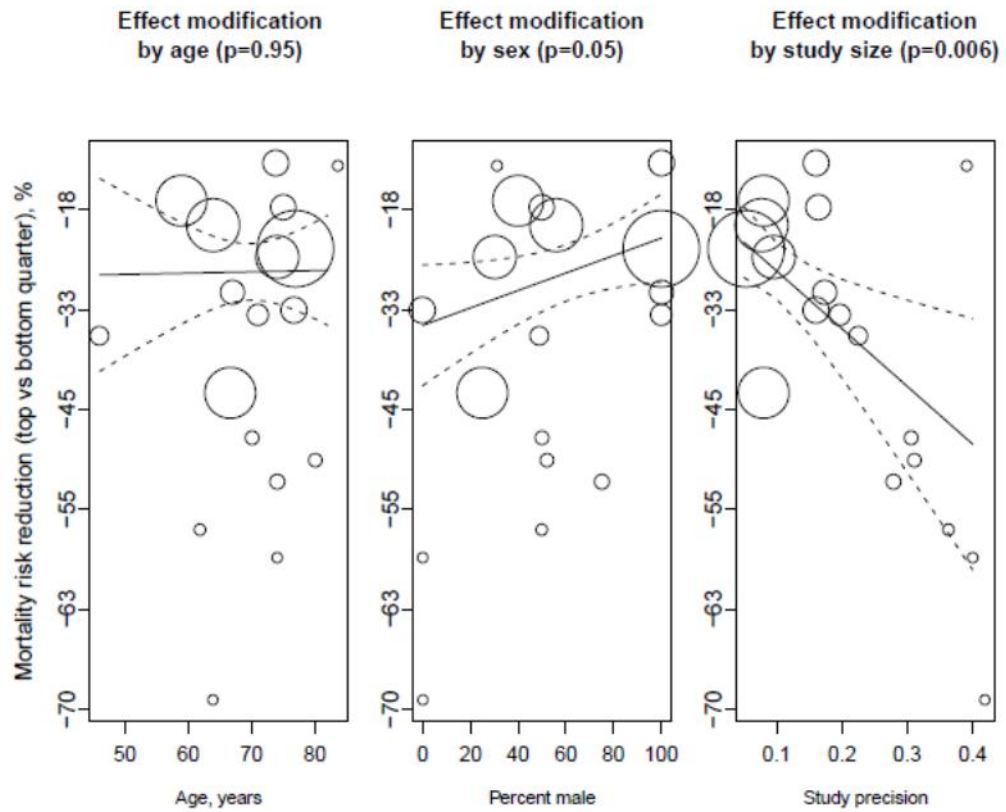


Figure 4-4: Meta-regression analyses of prospective studies reporting differences in 25(OH)D on risk of all-cause mortality, by age, sex and study size



After adjustment for age, percent male and study size, the p-values for effect modification are: age (p=0.42); male sex (p=0.12); study size (p=0.02)

Table 4-1: Summary characteristics of studies included in the meta-analyses of prospective studies

| Author, Year | Population characteristics | Country | N in study | Mean Age | Follow-up years | Endpoints | Number of events | | Level of adjustment ^a |
|---------------------------------|---|---------|------------|----------|-----------------|---|------------------|---------------|----------------------------------|
| | | | | | | | Total mortality | CVD mortality | |
| Anderson, ¹²⁸ 2010 | Intermountain Healthcare | US | 27686 | 66.6 | 1.3 | All-cause mortality | 1193 | - | 2+ S |
| Cawthon, ¹⁹⁶ 2010 | Osteoporotic fractures in Men (MrOS) study. | US | 1490 | 73.8 | 7.3 | All-cause mortality | 330 | 110 | + S |
| Dobnig, ¹⁹⁷ 2008 | LURIC study - The Ludwigshafen Risk and Cardiovascular Health study. | Germany | 3258 | 63.7 | 7.7 | / CVD mortality All-cause mortality / CVD mortality | 737 | 463 | 3+ S |
| Ensrud, ¹⁹⁸ 2010 | Women in the study of osteoporotic fractures (SOF) | US | 4551 | 76.7 | 4.5 | All-cause mortality | 432 | - | 2+S |
| Ford, ¹⁹⁹ 2011 | Third National Health and Nutrition Examination Survey (NHANES) mortality study | US | 7531 | 46 | 3.8 | All-cause mortality | 347 | - | 2+S |
| Hutchinson, ¹³³ 2010 | TROMSO study | Norway | 7161 | 58.9 | 11.7 | All-cause mortality / CVD mortality | 1359 | 513 | 3+ S |
| Jassal, ²⁰² 2010 | The Ranch Bernardo study. | US | 1073 | 74 | 6.4 | CVD mortality | - | 111 | 2+S |
| Jia, ²⁰³ 2007 | Aberdeen Community Health Index. | UK | 399 | 80 | 5.7 | All-cause mortality | 129 | - | 2+S |
| Kestenbaum, ²⁴⁹ 2011 | Cardiovascular health study | US | 2312 | 74 | 14 | All-cause mortality / CVD mortality | 1226 | 389 | 1+S |
| Kilkinen, ¹¹² 2009 | Mini-Finland Health survey | Finland | 6219 | 49.4 | 27.1 | CVD mortality | - | 933 | 2+ S |

Meta-analyses of prospective studies of vitamin D

| | | | | | | | | | |
|-----------------------------------|--|-------------|-------|-------|------|-------------------------------------|------|------|------|
| Kitamura, ²⁰⁴ 2010 | Yamato study | Japan | 205 | 83.6 | 2 | All-cause mortality | 42 | - | 3+ |
| Kuroda, ²⁰⁵ 2009 | Japanese postmenopausal women. | Japan | 1232 | 63.9 | 6.9 | All-cause mortality | 107 | - | 1+ |
| Melamed, ¹⁰⁷ 2005 | Third National Health and Nutrition Examination Survey | US | 13331 | 63.9 | 8.7 | All-cause mortality / CVD mortality | 1806 | 777 | 3+ S |
| Michaelsson, ¹⁴⁰ 2010* | Uppsala Longitudinal Study of Adult Men (ULSAM) | Sweden | 1075 | 71 | 12.7 | All-cause mortality / CVD mortality | 520 | 159 | 3+ S |
| Pilz, ²⁰⁷ 2009 | Hoorn study cohort. | Netherlands | 614 | 70 | 6.2 | All-cause mortality / CVD mortality | 51 | 20 | 3+ S |
| Semba, ²¹⁰ 2010 | 'Aging in the Chianti Area' (InCHIANTI) study. | Italy | 1006 | 74 | 6.5 | All-cause mortality / CVD mortality | 228 | 107 | 3+ S |
| Semba, ²¹¹ 2009 | Womens health and aging studies I and II. | US | 714 | 70-79 | 6.5 | All-cause mortality | 100 | - | 3+S |
| Szulc, ²¹² 2009 | MINOS study. | France | 782 | 67 | 10 | All-cause mortality | 182 | - | 2+S |
| Virtanen, ²¹⁴ 2010 | Kuopio IHD risk factor study (KIHD). | Finland | 1136 | 61.8 | 9.1 | All-cause mortality / CVD mortality | 87 | 35 | 3+ S |
| Visser, ²¹³ 2006 | Longitudinal Aging study Amsterdam (LASA). | Netherlands | 1260 | 55-85 | 6 | All-cause mortality | 380 | - | 3+S |
| Wang, ^{59**} 2008 | Framingham Offspring study | US | 1739 | 59.9 | 5.4 | *Incident CVD and CVD mortality | - | 120 | 2+ S |
| Tomson, ⁹ 2013 | Whitehall study. | UK | 5409 | 77 | 13 | All-cause mortality / CVD mortality | 3215 | 1358 | 3+ S |

* In this publication, patients in the lowest tenth of the 25(OH)D distribution were compared with patients between the 10th and 90th centiles. The numbers shown therefore exclude patients in the top tenth of the 25(OH)D distribution. ** This study reported a composite vascular outcome of vascular death or non-fatal vascular event (MI, angina coronary insufficiency, stroke, TIA, claudication or heart failure). α Level of adjustment: Age only +; Age AND at least 1 vascular risk factor (hypertension, diabetes, cholesterol) ++ Above AND a marker of physical status or social class +++. If adjusted for season or standardised for month/season of sampling = S.

Table 4-2: Quality assessment of the prospective studies vitamin D and mortality included in the meta-analyses

| Author | Study | Follow-up in years | Representativeness | CVD/mortality focussed outcomes | Factors included in the adjustment model* |
|-------------------|--|--------------------|--------------------|---------------------------------|---|
| Hutchinson, 2010 | TROMSO study | 11.7 | Yes | Y | 13589 |
| Virtanen, 2010 | Kuopio IHD risk factor study (KIHD). | 9.1 | Yes | Y | 123456 |
| Kuroda, 2009 | Japanese postmenopausal women. | 6.9 | Somewhat | Y | 18 |
| Melamed, 2005 | Third National Health and Nutrition Examination Survey (NHANES III). | 8.7 | Yes | Y | 123456789 |
| Anderson, 2010 | Intermountain Healthcare | 1.3 | Yes | Y | 12345 |
| Szulc, 2009 | MINOS study. | 10 | Somewhat | Y | 12568 |
| Pilz, 2009 | Hoorn study cohort. | 6.2 | Yes | Y | 12345689 |
| Michaelsson, 2010 | Uppsala Longitudinal Study of Adult Men (ULSAM) | 12.7 | Somewhat | Y | 12678 |
| Cawthon, 2010 | Osteoporotic fractures in Men (MrOS) study. | 7.3 | Somewhat | Y | 12 |
| Kestenbaum, 2011 | Cardiovascular health study | 14 | Yes | Y | |
| Semba, 2010 | 'Aging in the Chianti Area' (InCHIANTI) study. | 6.5 | Yes | Y | 1234568 |
| Semba, 2009 | Womens health and aging studies I and II. | 6.5 | Somewhat | Y | 1234568 race |

Meta-analyses of prospective studies of vitamin D

| | | | | | |
|----------------|--|-----|----------|----|----------|
| Visser, 2006 | Longitudinal Aging study Amsterdam (LASA). | 6 | Yes | Y | 1689 |
| Ensrud, 2010 | Women in the study of osteoporotic fractures (SOF) | 4.5 | Somewhat | No | 12678 |
| Tomson, 2013 | Whitehall study. | 13 | Somewhat | Y | 12345679 |
| Jia, 2007 | Aberdeen Community Health Index. | 5.7 | Yes | Y | 1258 |
| Kitamura, 2010 | Yamato study | 2 | Somewhat | No | 18 |

*Adjusted factors indicated by a digit: Age / sex = 1; Season = 2; HTN = 3; lipids = 4; DM = 5; Smoking = 6; Socio-economic status = 7; Physical activity = 8; renal function = 9; Representativeness was based on the authors' analysis whether the findings were generalisable to an age-matched general population based on participants included, their characteristics and outcomes evaluated

Table 4-3: Hazard ratios for all-cause mortality when studies were stratified by age

| Author, year | HR | CI lower | CI upper | Mean age |
|-------------------|------|----------|----------|----------|
| Hutchinson, 2010 | 0.83 | 0.68 | 1.02 | 58.9 |
| Virtanen, 2010 | 0.43 | 0.17 | 1.10 | 61.8 |
| Kuroda, 2009 | 0.31 | 0.10 | 0.90 | 63.9 |
| Melamed, 2005 | 0.64 | 0.36 | 1.14 | 63.9 |
| Anderson, 2010 | 0.57 | 0.46 | 0.70 | 66.6 |
| Szulc, 2009 | 0.69 | 0.44 | 1.08 | 67 |
| Pilz, 2009 | 0.52 | 0.24 | 1.14 | 70 |
| Michaelsson, 2010 | 0.66 | 0.40 | 1.10 | 71 |
| Cawthon, 2010 | 0.90 | 0.60 | 1.36 | 73.8 |
| Kestenbaum, 2011 | 0.74 | 0.58 | 0.95 | 74 |
| Semba, 2010 | 0.47 | 0.23 | 0.97 | 74 |
| Semba, 2009 | 0.41 | 0.15 | 1.14 | 74 |
| Visser, 2006 | 0.82 | 0.54 | 1.26 | 75 |
| Ensrud, 2010 | 0.67 | 0.44 | 1.01 | 76.7 |
| Tomson, 2013 | 0.76 | 0.66 | 0.87 | 77 |
| Jia, 2007 | 0.50 | 0.22 | 1.10 | 80 |
| Kitamura, 2010 | 0.89 | 0.33 | 2.44 | 83.6 |

Prospective studies ordered by increasing mean age of participants; HRs are for top versus bottom quartiles.

Table 4-4: Hazard ratios for all-cause mortality when studies were stratified by gender

| Author, year | HR | CI lower | CI upper | Mean age | Total participants | Doubling 25(OH)D between quartiles | Males% |
|--------------------------------------|-------------|-------------|-------------|----------|--------------------|------------------------------------|--------|
| Kuroda, 2009 | 0.31 | 0.1 | 0.9 | 63.9 | 1232 | N | 0 |
| Semba, 2009 | 0.41 | 0.15 | 1.14 | 74 | 714 | N | 0 |
| Ensrud, 2010 | 0.67 | 0.44 | 1.01 | 76.7 | 4551 | N | 0 |
| Studies in women | 0.46 | 0.23 | 1.02 | | | | |
| Anderson, 2010 | 0.57 | 0.46 | 0.7 | 66.6 | 27686 | Y | 25 |
| Kestenbaum, 2011 | 0.74 | 0.58 | 0.95 | 74 | 2312 | Y | 30 |
| Kitamura, 2010 | 0.89 | 0.33 | 2.44 | 83.6 | 205 | N | 31 |
| Hutchinson, 2010 | 0.83 | 0.68 | 1.02 | 58.9 | 7161 | N | 40 |
| Virtanen, 2010 | 0.43 | 0.17 | 1.1 | 61.8 | 1136 | Y | 50 |
| Pilz, 2009 | 0.52 | 0.24 | 1.14 | 70 | 614 | N | 50 |
| Visser, 2006 | 0.82 | 0.54 | 1.26 | 75 | 1260 | Y | 50 |
| Jia, 2007 | 0.5 | 0.22 | 1.1 | 80 | 399 | Y | 52 |
| Melamed, 2005 | 0.64 | 0.36 | 1.14 | 63.9 | 13331 | N | 56 |
| Semba, 2010 | 0.47 | 0.23 | 0.97 | 74 | 1006 | Y | 75 |
| Studies in a mixed population | 0.64 | 0.38 | 1.18 | | | | |
| Szulc, 2009 | 0.69 | 0.44 | 1.08 | 67 | 782 | N | 100 |
| Michaelsson, 2010* | 0.66 | 0.4 | 1.1 | 71 | 1075 | N | 100 |
| Cawthon, 2010 | 0.9 | 0.6 | 1.36 | 73.8 | 1490 | N | 100 |
| Tomson, 2013 | 0.76 | 0.66 | 0.87 | 77 | 5409 | N | 100 |
| Studies in men | 0.75 | 0.53 | 1.1 | | | | |

Chapter 5 : The Biochemical Efficacy and Safety Trial of vitamin D (BEST-D) - design and methods to evaluate effects of vitamin D on blood pressure and arterial stiffness

Introduction

This chapter describes the design and methods of a dose-finding trial of vitamin D, called the Biochemical Efficacy and Safety Trial of vitamin D (BEST-D). The trial design was also used to evaluate the effects of supplementation with vitamin D on blood pressure and arterial stiffness, and contributed to the work presented in Chapters 5 and 6 (Trial protocol published is in **Appendix 3**). The methods used to evaluate effects on blood pressure and arterial stiffness are included in this chapter.

Overview

Rationale and Aims of the BEST-D trial

About half of all women and a fifth of all men over the age of 50 years will experience a fracture in their lifetime and most will be related to the osteoporosis. There is uncertainty about whether supplementation with vitamin D for people with moderate vitamin D insufficiency can prevent osteoporotic fractures or other disease outcomes. Low circulating vitamin D levels in the population may be easily modified by supplementation with vitamin D.

In the United Kingdom, low circulating vitamin D levels are due to the combination of poor vitamin D in the diet and the lack of adequate sunlight-mediated production

of the vitamin.²⁵⁰ Older people have higher risks of developing fractures and falls, and are also at higher risk of developing CVD compared to the young. Hence, if supplementation with vitamin D could modify risks of CVD, fracture or falls, the absolute benefits would be greatest in the elderly.

Plasma 25(OH)D levels >90 nmol/L are typically found in young British adults at the end of the summer.²⁵¹ Evidence from the Whitehall resurvey demonstrated that optimal plasma levels of 25(OH)D for both CVD and non-CVD mortality were around 90 nmol/L.⁹ Even higher levels (about 115 nmol/L) are observed among people living closer to the equator.²⁵² Mean levels of plasma 25(OH)D among the UK resident population is around 55 nmol/L and to achieve plasma 25(OH)D levels of 90 nmol/L or more, the vitamin D supplement dose required to raise plasma levels to those seen at the end of summer will be at least 2000 IU daily. In order to maintain such higher concentrations (>90 nmol/L) throughout the year including winter, especially among the elderly, where plasma levels are lower than those observed in young people, even higher doses of vitamin D supplements of up to 4000 IU daily may be required. However, the safety and efficacy of taking such higher daily doses of vitamin D supplements in the older people is largely unknown.

The main aims of the Biochemical Efficacy and Safety Trial of vitamin D (BEST-D) were to compare the effects of daily supplementation with either 4000 IU vitamin D or 2000 IU vitamin D or placebo on biochemical markers of vitamin D status to determine the optimum dose to use in a large randomised trial of vitamin D

supplements evaluating effects on multiple health outcomes in older people living in the United Kingdom.

The trial was also used to investigate the effects of vitamin D on cardiovascular risk factors including assessment of the effects of vitamin D on blood pressure and arterial stiffness which are included in this thesis.

Rationale for evaluating effects of vitamin D on blood pressure

As discussed in Chapter 2, there is considerable uncertainty about whether taking vitamin D supplements may lower blood pressure. Most of the available randomised evidence comes from trials that used ‘insufficient doses’ of vitamin D supplements and are unlikely to have achieved optimal plasma 25(OH)D levels. Moreover, many trials had short periods of follow-up and small number of participants. There are very limited available data on whether higher doses of vitamin D supplements, ranging between 2000 IU to 4000 IU daily when taken daily for 1 year, could help reduce blood pressure favourably.

Rationale for evaluating effects of vitamin D on arterial stiffness

Arterial stiffness is a surrogate marker of CVD^{120, 253} and is a measure of arterial distensibility (compliance). Increased arterial stiffness is associated with adverse levels of other CVD risk factors and is an independent predictor of CVD events and all-cause mortality.^{121, 122, 254} Arterial stiffness may be modified and these measures

have been used as surrogate markers of CVD in clinical trials of risk factor modification therapy.²⁵⁵

Previous studies have reported that low vitamin D status is associated with an increased arterial stiffness (Chapter 2).¹²⁴ Data from 198 men living in the UK indicated that plasma 25(OH)D levels were inversely correlated with central aortic pulse wave velocity (a measure of arterial stiffness).^{163, 256} As discussed in Chapter 2, the association between low vitamin D and increased arterial stiffness could be linked to the vitamin D regulation of calcium metabolism, where low vitamin D may promote increased vascular calcification and, hence, the increased stiffening of arteries. Low vitamin D could also influence arterial stiffness indirectly, through effects on other CVD risk factors (such as blood pressure), or through pathways independent of the other known CVD risk factors. However, whether low vitamin D causes increased arterial stiffness is unproven and as these observed associations could be confounded. Further testing of the effects of vitamin D in a randomised-controlled clinical trial is required to ascertain causality. Further, it is unknown if arterial stiffness could be modified. If arterial stiffness were modifiable by administering vitamin D supplements; this could provide indirect evidence on the potential effects of vitamin D supplements on CVD outcomes, and help in the interpretation of planned or on-going trials of vitamin D.

Arterial pulse wave analysis for arterial stiffness

The 'arterial pulse wave' is a composite of a forward wave, formed after the contractile force of the heart (or propagative wave) and a retrograde 'reflected wave,' (**Figure 5-1**), caused by the reflection of the forward wave from points further along the arterial tree, typically at the major branches of the arteries (e.g. bifurcation of the descending aorta).^{257, 258} The inflection point, where the forward and reflected waves meet, depends on the extent of stiffness of the arterial wall and influences the velocity of propagation of the pulse wave. When arteries stiffen, the pulse wave velocity (PWV) increases, resulting in faster wave transmission (see **Figure 5-1** for explanation). It is thought that the reflected wave helps 'augment' the central aortic blood pressure (causing a higher central pressure) when compared to the pressure in the peripheral arteries (for instance the brachial artery).²⁵⁹

Selecting methods and indices of arterial stiffness for use in the BEST-D

Measurement of arterial stiffness and the nature of the pulse is complex.²⁶⁰ Several indices (**Table 5-1**) have been derived using different methods to quantify changes in the properties of the arterial wall stiffness (reviewed in detail elsewhere^{161, 257, 261}). Aortic stiffness, commonly measured by estimating pulse-wave velocity, is considered the gold standard. Typically, pulse wave velocity is estimated by measuring the carotid-femoral pulse wave velocity, and is estimated by the time difference for the pulse wave reaching the carotid artery in the neck and the femoral artery in the groin (with the latter typically being delayed, reflecting the

speed of propagation of the wave). This measurement is, however, commonly conducted in a controlled-laboratory setting, with the participant in the recumbent position, and can be affected by factors such as caffeine and sleep deprivation,²⁶² and assessments recorded by a trained physiologist. Hence, this method was deemed unsuitable for use in the BEST-D trial which involved a community-based study of older people, with planned home visits.

Newer methods of assessing arterial stiffness have recently been developed, mainly based on 'pulse wave analyses' where the pulse wave velocity is estimated indirectly from the analysis of the pulse wave morphology recorded at the peripheries (typically limb arteries).²⁶³ This commonly involves recording the pulse wave at a peripheral artery. The most widely used method to assess arterial stiffness is 'radial artery applanation tonometry', which estimates the pulse wave velocity by calculating the time taken for the reflected and forward waves to meet (**Figure 5-1**) based on the analyses of the recorded waves. However, many of these methods lack adequate validation studies (although such validation may emerge in future studies) and such methods lack wider acceptance and use in clinical practice or research.

Another problem is the lack of comparability of different measures of arterial stiffness recorded at different sites the body. Whether the measures recorded at different points of the arterial tree are truly comparable or interchangeable is unknown, and previous comparisons indicate poor agreement between sites. This is likely because central large artery wall structure is comprised of more muscular and

elastic layers than the thinner, narrower walls of the peripheral arteries and arterioles. In other words, the distensibility of the larger more proximal arteries may not be similar to those in the limb peripheries by virtue of differences in their structure and anatomy. Other local factors may also influence this variability. For example, the arterioles in the digits may respond to local temperature changes and blood volume changes more than that of the central arteries. However, emerging data indicate, that although the pulsewave-analyses derived measures are not interchangeable, these 'local stiffness measures' may be independently predictive of CVD risk and hence still useful to provide prognostic data.²⁶⁴

As participants in the BEST-D trial were older people, and the trial involved home visits undertaken by a study nurse, a suitable device for measuring arterial stiffness needed to be a portable, cost-effective, reliable, and simple-to-use method to record these measurements. Based on these considerations, novel methods estimating pulse wave velocity using peripheral limb arteries were deemed more appropriate and feasible than the central artery stiffness assessments. After evaluating the then available non-invasive methods for measuring arterial stiffness [radial tonometry, cardio-ankle vascular index (CAVI), brachial artery stiffness, digital pulse volume] in internal assessment pilots held at CTSU (Data not shown), technical, logistic and feasibility problems were identified with the radial tonometry and the CAVI assessment methods. For instance, 'applanation tonometry' required, adequate training of personnel (here a probe is held over the radial artery in the forearm to record a pulse wave), co-operation and patience from the participant who had to remain still during the recording, participant being recumbent, and

required repeated measurements before a 'valid trace' of adequate quality was obtained (Data not shown). The measurement of the cardio-ankle vascular index required the participants to remain recumbent and motionless during the duration of the measurement (for several minutes) and required a flat couch and a few minutes for preparation. Further, given the older participants in the BEST-D trial, frailty and presence of tremors were likely to be common and yielding poor quality traces. Hence, these methods were believed to be unsuitable for use in the BEST-D trial. The Tensiomed Arteriograph device and the Pulsetrace PCA II were believed to be more suitable and selected for use in the BEST-D trial.

Arteriograph

The Tensiomed Arteriograph (ARG) device (**Figure 5-2**)^{265, 266} was used to measure blood pressure and arterial stiffness from the right arm. The ARG records arterial pressure waveforms at the brachial artery using an 'oscillometric method'. The device consists of a battery powered motor attached to a cuff, similar to a blood pressure cuff, connected using a blue-tooth link to the study laptop. The initial cuff inflation records the blood pressure in a similar manner to an automated blood pressure machine. During a further inflation, the brachial blood flow is occluded by raising the cuff pressure to 35 mmHg above the recorded systolic pressure. By creating a 'stop-flow' condition, the upper edge of the cuff is able to detect the pressure changes (and record the pulse waves over a period of 8 seconds) at the upper arm. The pressure changes are recorded by a high fidelity pressure sensor and the signals transmitted using a blue-tooth link to a laptop. The device also

provides measures of blood pressure and heart rate. Two key measures were derived for arterial stiffness from the ARG:

(i) Pulse wave velocity (PWVao): The time taken for the forward wave and the reflected wave to meet, the return time (RT), was measured. The distance from the sternal notch to the upper edge of the pubic bone, the jugulo-symphisial distance (D), was used as an estimate of the distance from the aortic arch to the aortic bifurcation. The PWVao was calculated using the formula:

$$\text{PWVao (m/s)} = D / (\text{RT} / 2).$$

(ii) Augmentation Index (Alx): Augmentation index is an index of the augmentation of the systolic pressure, at the central aorta, thought to be caused by the reflection of the pulse wave. Alx is the augmentation pressure expressed as a percentage of central pulse pressure. Alx, thus reflects properties of both central aortic stiffness (distensibility) and that of wave reflection. The value also depends on the state of the peripheral arteries and up to the point of reflection of the forward wave. The ARG software uses the following formula to calculate Alx:

$\text{Alx} = \text{P2} - \text{P1} / \text{PP} \times 100$, where P1 is the amplitude of the forward wave, P2 is the amplitude of the reflected wave and PP is the pulse pressure.

These measurements were then recorded into the electronic case report. The software prompted a repeat reading, if the standard deviation of the recordings displayed as a SD value (this is automatically calculated by the ARG software as a measure of quality of the tracing, derived by comparing the waves recorded over an

8 second period) was >1 . If further attempts failed to record a suitable measurement, the study nurse was permitted to proceed with the rest of the visit, and omitting the reading (as in these instances, the recordings were less likely to be consistent or reproducible in our prior testing).

ARG device is validated for blood pressure and arterial stiffness.^{263, 267} Reported mean (SD) values for PWVao were 9.46 (1.8) m/s and for Aix were 30 (13), but these studies were predominantly conducted in younger people. In a UK study of 198 men (age range between 40-80 years), mean PWVao was 6.9 m/s and Aix was 33% among Caucasians.²⁵⁶ The portability of the device, the ease of use, the minimal training required and the relative operator-independent measurements were the main advantages for the ARG. The disadvantages included the lack of published reports on validation and prognostic information, and that the measurements were only indirect indicators of central aortic stiffness, where much of the data on arterial stiffness was derived from.

Pulsetrace PCA 2

The Pulsetrace PCA 2 (**Figure 5-2**) uses photoplethysmography (infrared) to record changes in the 'digital pulse volume' (DWP).²⁶⁸ A finger probe, similar to those used to measure pulse oximetry, was placed on the right forefinger (or middle finger). The infrared sensor senses the pulse wave morphology, but likely to be a composite trace of the local digital pressure and volume changes, rather than exclusively reflecting vascular wall stiffness. The device provided two measures from the pulse wave analysis (**Figure 5-3**):

- (i) Stiffness index (SI): defined as participant height / PPT, where PPT stands for the time between the peaks of the forward and reflected waves; and
- (ii) Reflective index (RI): defined as the height of the forward wave divided by the height of the reflected wave.

The Pulsetrace device also suffers from having very little published validation and prognostic data, with much of the published literature being from a single group. From these limited earlier studies, the mean (SD) SI for 60 year olds was around 12.2m/s and RI was 60 (5)% in a small sample; the range for SI was 6-10m/s (for all study participants; n=87; age 21-68 years)²⁶⁹ and for RI between 60-90%.²⁷⁰

The Pulsetrace PCA 2 device has been used in 100,000 people in the UK Biobank study²⁷¹ (which should provide normative and possibly prognostic data in future), but at present there is limited information. It is not known how comparable the digital volume pulse measurements could be with other measures of central arterial stiffness measurements, as the device records traces from the small digital arteries rather than the larger proximal or central arteries as assessed by other methods. Nevertheless, it could potentially provide useful information on local arterial pulse physiology, which may be equally of prognostic significance. As this method is operator-independent, highly portable, and requires minimal training, it was found to be suitable for use in the BEST-D trial.

Hypothesis and aims for the effects of vitamin D on blood pressure and arterial stiffness

The study tested the hypothesis that dietary supplementation with high dose vitamin D (4000 IU and 2000 IU) for 12 months had no effect on arterial stiffness and blood pressure.

The aims were to evaluate:

- (i) Effects of the 2 doses of vitamin D3 (2000 IU vs 4000 IU) versus placebo on blood pressure at 6 and 12 months of treatment;
- (ii) Effects of the 2 doses of vitamin D3 (2000 IU vs 4000 IU) versus placebo on arterial stiffness measured by the Arteriograph and Pulsetrace at 6 months.

Methods

BEST-D trial outline and study assessments

The BEST-D trial was a randomised, double-blind, placebo-controlled dose-finding trial of vitamin D3. Eligible participants (**Table 5-2**) were older people (>65 years) living in the community of Oxfordshire, and randomised using a 1:1:1 allocation to receive either 4000 IU, 2000 IU of vitamin D3 or matching placebo for 12 months (**Figure 5-4**). The trial protocol of the BEST-D trial was published (**Appendix 3**) and the Data Analysis Plan is included in (**Appendix 4**).

Study visits and assessments

Participant recruitment

Potential participants were identified from the medical records of the participating GP practice (Hightown surgery) in Banbury, Oxfordshire from among patients registered at the GP Practice. The Practice is based in Banbury, a market town in Oxfordshire, which had about 10,200 registered patients, and most patients lived in urban areas with only small proportion living in semi-rural areas. The vast majority of patients had Caucasian ancestry (minority were Asian or other ethnic populations), and had a similar age and gender distribution to the national average for UK GP practices. However, the population was less affluent than the overall UK population (index of multiple deprivation score: 12.5 vs 21.8). This practice became the single recruiting practice for the study as, (i) the GP surgery was the pilot site and had a previous record of conducting studies and had collaborated with CTSU before; and (ii) an initial search of the registered patients at the practice showed more than a thousand patients potentially meeting recruitment criteria for the trial; and (iii) the General Practitioner was one of the investigators and the site had adequate resources to accommodate the trial requirements for access and space; and finally, (iv) the recruitment targets were met before other NIHR sites were needed to be identified. Participants were invited by their general practitioner, Dr Harold Hin, the General Practitioner and local investigator for the study, to participate in the trial if they met eligibility criteria (**Table 5-2**). Patients were asked if they were willing to participate by sending them an invitation. The GP Practice sent invitations by mail which included an information leaflet, a response form and a freepost envelope. Potential participants showing intent to participate were

contacted by the receptionist and a brief 'Screening questionnaire' was completed to confirm willingness and suitability to participate in the trial. An appointment with the BEST-D study nurse was made to visit their homes at a suitable time.

Randomization Visit

At the first visit (Randomization Visit), the BEST-D study nurse visited the participants' homes to discuss study procedures and sought informed consent if participants were still willing to participate. After obtaining informed consent, relevant medical history and eligibility were recorded onto an electronic case report form, using a customized software programme loaded into a secure laptop. These included demographic details, history of CVD, assessment of physical activity levels, muscle and joint pains, risk factors for CVD, assessment of dietary calcium intake in the previous week using a calcium questionnaire (developed based on the Nelson's questionnaire²⁷²), and current use of prescribed or any over-the-counter medications. Anthropometric measurements included weight, height, arm circumference, and the sterno-symphysial distance. Blood pressure and arterial stiffness were measured using the Tensiomed Arteriograph and Pulsetrace PCA II devices. Blood samples were collected and stored at -80°C. Participants were offered information leaflets on good diet sources of calcium.

Eligible participants were randomised at this point by contacting the central coordinating office based at the CTSU via telephone. A treatment allocation number was provided after successful randomization, which when entered into the laptop enabled the double-blind dispensing of appropriate treatment from the supplies of

vitamin D supplements or placebo provided. Follow-up visit appointments were provisionally made. The visit assessments were stream-lined to enable collection of the most relevant and useful information in an efficient and timely manner, with an average visit taking just over an hour for the randomization visit and about 45 minutes for the follow-up visits. In view of the older participants, visual aids were used to assist selecting appropriate responses with a visual analog scale and intake of dietary calcium assessments, to standardize the responses. A 4-point questionnaire was used to assess intake of dietary calcium, and to enable estimation, a visual aid depicting common portion sizes was used.

Follow-up visits

1 month:

In a randomly selected group of 100 participants, a blood sample was taken at 1 month after randomization.

Follow-up visits:

At the follow-up visits (**Table 5-3**), the study nurse recorded serious adverse events and changes to non-study medication at 6 and 12 months. Participants were also asked about self-reported physical activity and adherence with study medication, which was recorded as the participant having taken 'most', 'some', 'very little or none' of the study treatment. The reasons for discontinuation of any study treatment were recorded. The clinical assessments were broadly similar to those carried out at the randomization visit (see **Table 5-3**). At the 6 month visit, a

7-month supply of study medication and an appointment for the final visit were given. At the 12 month visit any unused treatment was collected.

Blood pressure and arterial stiffness assessments

At each visit all participants had measurements of their blood pressure and vascular stiffness recorded using the TensioClinic™ Arteriograph and the Pulsetrace PCA II devices (**Figure 5-2**). At the first visit only, height and weight (for BMI calculation) were recorded, together with mid-arm circumference, using a measuring tape, and the sterno-symphysial distance, using a bespoke wooden scale to record the linear distance between the anatomical landmarks between the sternal notch and the pubic symphysis. This was used as a surrogate measure of the length of the descending aorta, which reflects the distance travelled by the reflected pulse wave from the aortic bifurcation.

For recording of blood pressure and arterial stiffness, participants were asked to be seated comfortably in a chair (with arm rests or near a table) and after 10 minutes of rest, the finger probe of the Pulsetrace PCA 2 was placed on the right forefinger to record the pulse wave over a 30-60 second period. This was followed by measurements of blood pressure and arterial stiffness made by placing a cuff over the upper arm, and recordings taken over 2 minutes using the TensioClinic™® Arteriograph, the process being similar to a standard blood pressure assessment.

Whenever possible, the study visits were scheduled at the same time of the day to reduce the effects of diurnal variations in these measures. Participants were advised to avoid alcoholic beverages, tea and coffee before each visit.

Outcomes

Outcomes of the main BEST-D trial

The primary efficacy assessment was an “intention-to-treat” analysis among all randomised participants on the proportion of individuals with levels of 25(OH)D above 90 nmol/L at the end of the study in each treatment group. A co-primary endpoint was the difference between those allocated 100 vs 50 µg daily, in the mean 25(OH)D levels at the scheduled study end. The study also assessed a number of secondary and tertiary outcomes (**Table 5-4**) as shown in **Table 5-3**.

Blood pressure and arterial stiffness outcomes

The comparisons involved “intention-to-treat” analyses among all randomised subjects of the effects of daily vitamin D3 supplements 4000 IU vs placebo, vitamin D3 2000 IU vs placebo, and the difference between the two doses of vitamin D on:

- (i) The difference in the change from baseline in diastolic and systolic blood pressure and heart rate at 6 and 12 months.
- (ii) The difference in the change from baseline in arterial stiffness (pulse wave velocity, Augmentation Index, Stiffness index and reflective index) at 6 months.

Trial registration and approval

The trial was registered with the International Standard Randomised Controlled Trial Number Register (ISRCTN07034656). The trial protocol was approved by the NRES Committee South Central - Oxford B, and the study REC reference is 12/SC/0243. MHRA approved the study on 18th June 2012 (Ref 21584/0295/001-0001) and the trial EUDRACT Number is 2011-005763-24. BEST-D is included in the NIHR portfolio (reference CSP 67777) after receiving R&D approval on 13th August 2012 from the Thames Valley Primary Care Research Partnership (**Appendix 5**).

Statistical analyses

Statistical analyses were performed according to a Data Analysis Plan (**Appendix 4**) agreed by the trial Steering Committee prior to any unblinding of study results.¹⁷ The primary efficacy assessment for the main BEST-D trial was an “intention-to-treat” analysis among all randomised participants of daily supplementation with vitamin D3 4000 IU vs vitamin D3 2000 IU on the proportion of individuals with levels of 25(OH)D above 90 nmol/L at the end 1 year. A co-primary endpoint was the difference between those allocated 4000 IU versus 2000 IU daily in the mean 25(OH)D levels at the study end. If a 12-month value was not available, the 6-month value was used.

BEST-D trial

The main objective of the BEST-D trial was to find the dose of vitamin D3, that would achieve a 25(OH)D level of more than 90 nmol/L in at least 90% of the participants in the treatment arm. With 100 participants in each group, there was

>99% power at $2p < 0.01$ to detect a difference in the proportion of participants with a 12 month 25(OH)D level >90 nmol/L between those allocated 4000 IU and 2000 IU daily. With 100 in each treatment group any common adverse effects from administering high daily doses would have become apparent (i.e. the trial should be able to detect adverse events that have a 1% chance of occurring every year).

Power to detect effects on blood pressure and arterial stiffness

Developing a sample size and power calculation for the evaluation of blood pressure and arterial stiffness effects was not possible, as it was difficult to estimate differences with treatment as there was hardly any data for a similar vitamin D trial to compare at that time. However, we calculated that based on assumptions that if the trial were to detect a 10% difference in systolic blood pressure (and/or pulse wave velocity) from baseline values, at least 70 participants would need to be in the vitamin D treatment arm and placebo arms respectively, to have a >90% power to detect this change (α 0.05). The current trial design would therefore have had sufficient power, even when reasonable adjustments for adherence and attrition were to be taken into account. Finally, the VitDish trial recruited fewer than 200 participants in a contemporary trial of vitamin D therapy,¹⁷⁷ and many other trials evaluating effects on blood pressure included even fewer participants. The current study would be the largest and longest study to test the effects of taking either 2000 IU or 4000 IU vitamin D daily, on arterial stiffness.

Allowance for multiple comparisons

No allowance was made for multiple comparisons in the assessment of the primary endpoints and a two sided p value of <0.05 was considered significant. For the secondary and tertiary analyses, allowance was made in their interpretation for multiple hypotheses testing taking into account, if relevant, the type of measure and evidence from other studies. But, the more extreme the p-value (or, analogously, the further the lower limit of the confidence interval is from zero) after any allowance has been made for the nature of the particular comparison, the more reliable the comparison and, hence, the more definite any finding was considered to be. Data are presented as means and SD. Differences between the paired measures at baseline and 12 months were compared using t-tests.

Study treatment

Participants were randomly allocated to receive either 4000 IU or 2000 IU vitamin D3 or matching placebo daily in a double-blind manner. The nurse telephoned CTSU, and provided relevant details (as a summary code generated by the computer program) which was used in a minimisation algorithm balanced for age group (<70 , 70 to <75 , ≥ 75 years), gender, smoking history (current versus ex or non-smoker), ethnicity (Caucasian vs non-Caucasian), BMI (<25 , 25 to <30 , ≥ 30 kg/m²) and history of fracture (yes, no) to allocate participants to one of the three treatment arms. The allocation to treatment was also conveyed as an electronic code (blinded), which when entered into the bespoke BEST-D software (Edgehill) in

the laptop, was used to allocate treatment from the supplies carried by the nurse (through an in-built treatment dispensing module of the programme).

Participants were given two treatment containers labelled differently, and asked to take one capsule from each container. Each container contained 210 capsules of either vitamin D3 (2000 IU) or placebo, sufficient to last 7 months. The varying combination of these containers facilitated appropriate treatment allocations in the three arms. The soft gel capsules were provided free of charge by Tischcon Corporation, Westbury, New York, USA. They were packaged in child-proof containers by Bilcare GCS (Europe) Ltd. The study nurses were responsible for appropriate storage, dispensing and documented disposal of unused and returned medications.

Assessment of adherence with treatment

Treatment adherence was evaluated by the study nurse at the 6 and 12 month follow-up by indicating whether the participant had taken *Most, Some, Very little or none* of the study treatment. The participants were also asked if they had missed any treatment in the last 7 days and to indicate how many days of treatment were missed. Biochemical safety was evaluated by the effects of vitamin D3 supplements on plasma PTH, calcium and phosphate levels.

Blood samples

Blood collection

Blood samples were collected into a 10ml EDTA tube and a 10 ml Lithium Heparin tube. In addition samples were collected at baseline and 12 months for future mRNA and DNA analyses. All participants with hypercalcaemia at baseline were excluded.

Blood transport

Samples were transported in an ice-cooled 'blood sample box' prepared with ice packs, and taken to the Horton Hospital Clinical Chemistry laboratory. The plasma samples were centrifuged for 10 minutes at 1200g before being fractionated into aliquots and stored in a -80°C freezer. Samples were shipped periodically by courier using dry ice and with appropriate precautions for handling clinical hazardous materials, to the Wolfson laboratory in CTSU, Oxford for long-term storage and future analyses.

Assays

Blood was sent for assays as outlined in the protocol (**Appendix 3**). Only the relevant results of plasma 25(OH)D concentrations, as a response to vitamin D3 supplements, are included in this thesis.

Safety monitoring

Vitamin D toxicity

Vitamin D toxicity could cause hypercalcaemia which may present with symptoms such as constipation, diarrhea, decreased appetite (anorexia), dehydration, polyuria, thirst, vertigo, fatigue, bone pain, muscle weakness, irritability, abdominal pain, nausea, vomiting, mental confusion, impaired renal function, kidney stones, and cardiac arrhythmias.²⁷³ However, there are no recognized adverse effects associated with 25(OH)D levels below 240 nmol/L^{92, 274} and previous reports of vitamin D toxicity with elevated serum calcium have only been observed with very high intake of vitamin D ≥ 40000 IU (1000 μ g) a day and at blood 25(OH)D levels > 200-300 nmol/L.

Adverse events

Non-serious adverse events

Only adverse events not thought to be serious or related to the study treatment, but resulting in the participants stopping treatment were recorded at each follow-up as non-serious adverse events. All other non-serious adverse events were not recorded as such events are common in this age group, and analysis of such events is unlikely to yield any useful information.

Serious Adverse Events

Serious Adverse Events (SAE) were any adverse events that were thought to be serious (causing death, disability, or hospitalization), and were recorded and

reported to regulatory authorities as appropriate via the annual Drug Safety Update Report (DSUR). Serious adverse events were defined as those adverse events that:

- resulted in death;
- were life-threatening;
- required in-patient hospitalization or prolongation of existing hospitalization;
- resulted in persistent or significant disability or incapacity;
- resulted in congenital anomaly or birth defect;
- were important medical events in the opinion of the responsible investigator including, for the present study hypercalcaemia, kidney stones and cancer. The normal serum calcium levels were considered to be between 2.15-2.55 mmol/L, based on laboratory reference ranges.

The clinical investigator team had to ensure that all serious adverse events were reviewed as soon as possible and was classified as either 'related' or 'unrelated' to study treatment.

Safety Data Monitor

Any suspected serious adverse reactions (SSAR) were to be reviewed by the independent Safety Data Monitor (Professor Colin Baigent, CTSU, University of Oxford) who was responsible for making relevant recommendations to the Steering Committee after review.

Data collection

All data collected were recorded in electronic format on bespoke trial software (called Edgehill) developed by the CTSU IT team and the clinicians. The study laptops used by the study nurses were securely encrypted. Stored data were periodically backed-up electronically to the CTSU servers at University of Oxford.

Study procedures and training

The study team members were fully trained on all aspects of the trial. I was responsible for the design, creation and delivery of the training sessions for BEST-D trial procedures. The training sessions were held at CTSU, University of Oxford (Richard Doll Building, Roosevelt Drive). Training was provided at the start and before every phase of the study (e.g. 6 month visit, final visit etc). Training included presentations, printed training materials, relevant SOPs, training in laboratory methods, and hands-on training of trial equipment and software. If further support was necessary, this was provided by relevant study team members when required, either by phone or at the local surgery in Banbury. Contact details were also provided for emergency contact in case of technical or medical problems, and the study nurses were encouraged to be in contact as often as needed, either by email (bestd@ctsu.ox.ac.uk) or by telephone to the CTSU study team.

Conclusion

The design of the BEST-D trial, and the state-of-the-art methods used to evaluate the effects of vitamin D supplements on blood pressure and arterial stiffness should maximize the chance of detection of beneficial effects of vitamin D on blood pressure and on arterial stiffness. The results of the BEST-D trial are presented in the next chapter.

Chapter 5: Figures and Tables

Figure 5-1: Pulse wave morphology and pulse wave analysis

In a normal pulse wave, the first 'forward' or 'propagated' wave is caused by the contractile pushing force of the left ventricle. The notch marks the 'inflection point' where the reflected wave meets the forward systolic wave; these reflected waves arise as a result of reflections of the forward waves at arterial bifurcation points. The velocities of the forward and reflected waves increase when there is increased vessel wall stiffness. This translates into a shift of the inflection point. When arterial stiffness increases, the inflection point occurs earlier (shifts to the left in the diagram; it can also disappear entirely in extremely stiffened arteries).

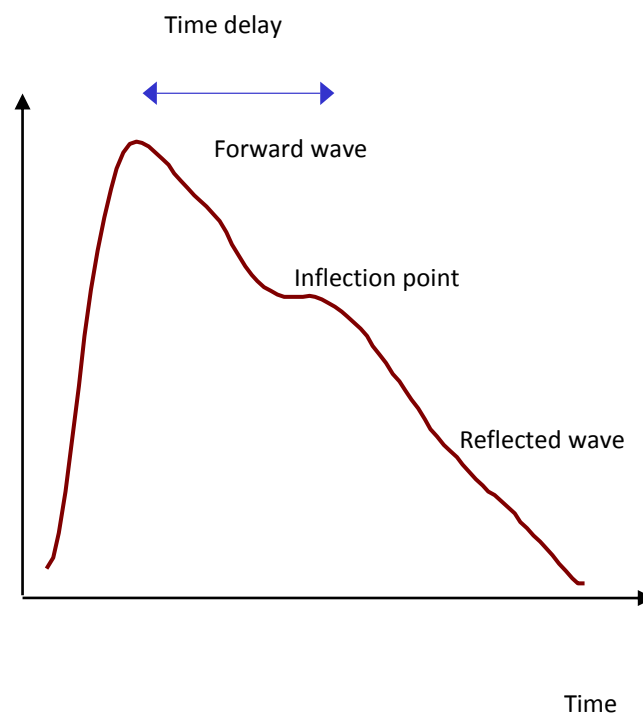


Figure 5-2: Tensiomed Arteriograph and Pulsetrace PCA II

Arteriograph



Pulsetrace PCA II



Figure 5-3: Pulse wave analysis by the Pulsetrace PCA 2 (from device information leaflet)

The calculations of the SI and RI from the pulse wave morphology are shown below.

PPT is the time between the two peaks 'the forward wave' and 'the reflected wave'.

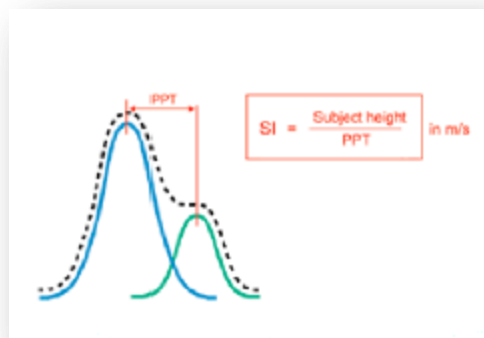


Figure 5-4: BEST-D trial outline

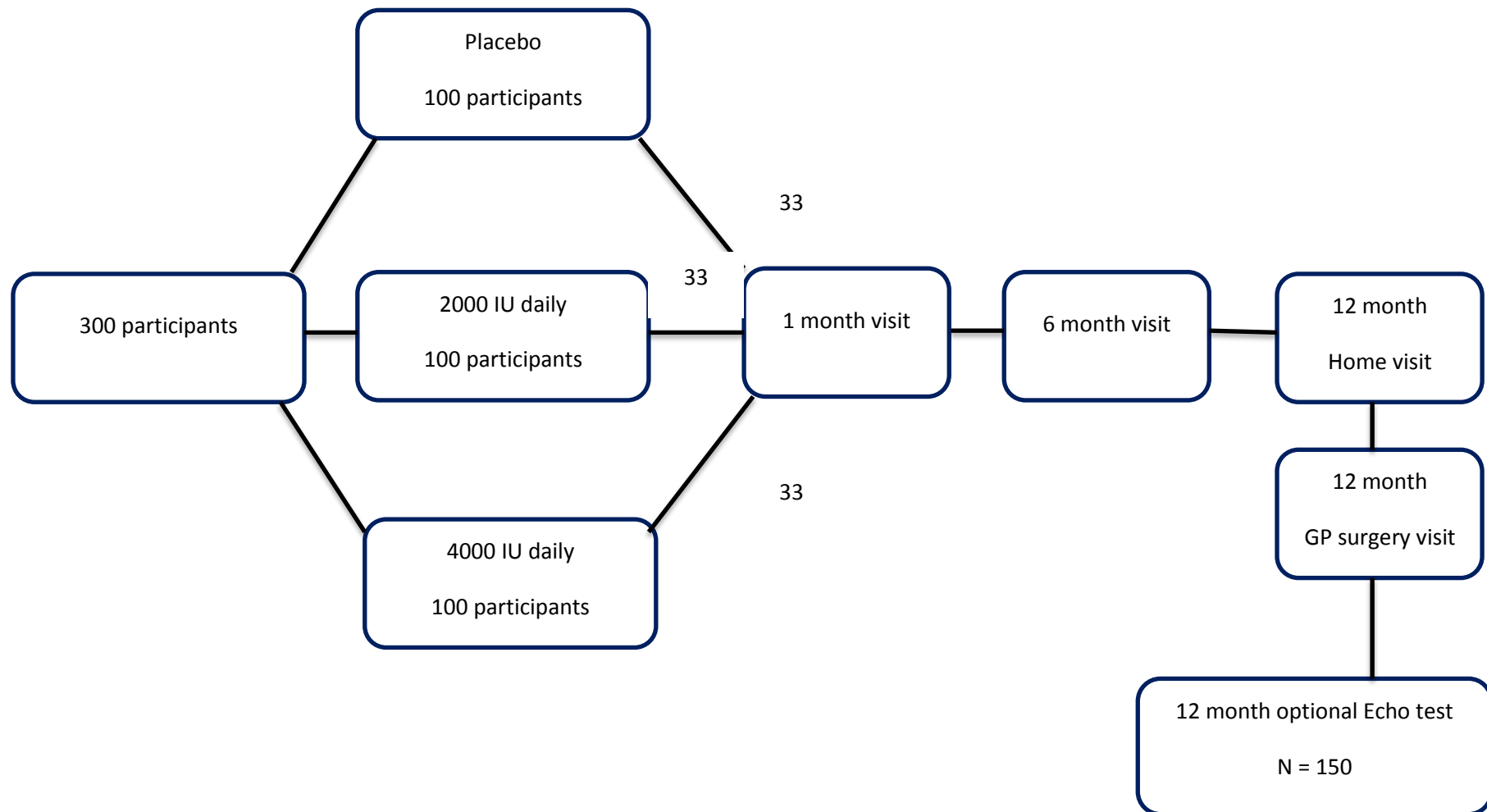


Table 5-1: Comparison of different measures of arterial stiffness

| Measure | Definition | Formula |
|---------------------|--|-------------------------------------|
| Compliance | The absolute change in vessel diameter for a given change in pressure | dD / dP |
| Distensibility | The relative change in vessel wall diameter for a change in pressure | $dD \text{ (or } dA) / dP \times D$ |
| Elastic modulus | This is the inverse of distensibility | $dP \times D / dD \text{ (or } dA)$ |
| Pulse wave velocity | The speed with which the pulse wave travels between two points in a vessel | L/t |
| Stiffness index | This is a ratio of the natural log of systolic blood pressure to diastolic blood pressure relative to the change in diameter | $\ln (SBP/DBP) / (D2-D1/D2)$ |
| Young's modulus | Elastic modulus per unit area of vessel wall | $dP \times D / dD \times t$ |

dD – change in diameter; dP – change in blood pressure; dA – change in vessel area; L-Distance between 2 points in the vessel; t – time; Ln – natural log; D1 and D2 – initial diameter, and subsequent diameter.

Table 5-2: Eligibility criteria for BEST-D: Patients were eligible if they meet all inclusion criteria, and if no exclusion criterion applies.

| Inclusion criteria | Exclusion criteria |
|---|--|
| <ul style="list-style-type: none"> - Age \geq 65 years - Living in the community - Ambulatory | <ul style="list-style-type: none"> - Nursing home residents - Regular use of vitamin D supplements with >400 IU (10 μg) vitamin D daily - Use of alendronate, risedronate, zoledronic acid, parathyroid hormone, or calcitonin - Medically diagnosed dementia - History of hypercalcaemia, hyperparathyroidism, lymphoma, sarcoidosis, active tuberculosis - History of renal calculus - Known to be poorly compliant with clinic visits or with taking medication - Recent history of alcohol or substance misuse or abuse - Medical history that might limit the ability of the subject to take the study treatment for the duration of the study (e.g. terminal illness) - Regular prescribed calcium supplements. |

Table 5-3: Summary of BEST-D trial procedures

| Month | 0 | 1 | 6 | 12 |
|--|---|----|---|----|
| Relevant medical history and assessment of eligibility | X | | | |
| Informed consent if willing and eligible | X | | | |
| Assessment of dietary calcium | X | | | |
| Height and weight | X | | | X |
| Blood pressure sitting, pulse wave velocity (brachial and digital arterial stiffness) | X | | X | X |
| Hand-grip muscle strength and muscle pain | X | | X | X |
| Assessment of fractures (no., site, date) and falls (no., date) | | | X | X |
| Geriatric Depression Score (4 point) | | | X | X |
| Heel-bone and wrist DEXA measured ^π | | | | X |
| Short Physical Performance battery ^π | | | | X |
| Blood sample taken for plasma 25(OH)D, PTH,* calcium, phosphate, albumin, alkaline phosphatase, creatinine and lipids† | X | X* | X | X |
| hsCRP and nBNP and other markers of inflammation. | X | | X | X |
| Buffy coat for genetic studies | X | | | X |
| PAXgene Blood RNA tube for mRNA expression | X | | | X |
| Store plasma for future assays‡ | X | | X | X |
| Issue study treatment | X | | X | |
| Assessment of adherence | | | X | X |
| Serious adverse events and non-serious AEs due to study treatment cessation will be reported | | | X | X |

*25(OH)D and PTH measured at 1 mo in a subgroup of 100 patients stratified by allocated treatment. Only blood collection will be carried out at the 1 month. † Lipid profile includes total cholesterol, LDL-C, HDL-C, triglycerides, apolipoprotein A, apolipoprotein B.

‡ Plasma stored for assays of bone markers, cytokines and RNA markers of inflammation (when additional funds are available). 25-hydroxy-vitaminD (25[OH]D); parathyroid hormone; (PTH), C-reactive protein (CRP); n-terminal brain natriuretic peptide (nBNP). π All participants will be offered an appointment for these assessment to be done at the Hightown Surgery.

Table 5-4: Secondary and Tertiary outcomes in BEST-D

Secondary outcomes

All secondary assessments will involve “intention-to-treat” analyses among all randomised subjects of the effects of daily supplementation with vitamin D3 100µg vs placebo, vitamin D3 50µg vs placebo, and the difference between the two doses of vitamin D on:

- (i) Mean blood levels of 25(OH)D during follow-up.
- (ii) The proportions of participants with blood 25(OH)D levels >90 nmol/L at the 6 and 12 month visits.
- (iii) The proportion of participants with PTH levels suppressed into the normal range (1.1-6.8 pmol/L) at the 6 and 12 month visits.
- (iv) The proportion of participants with calcium levels above the normal range (2.15-2.55 mmol/L) at the 6 and 12 month visits.
- (v) Other laboratory tests of safety: phosphate, albumin, creatinine, alkaline phosphatase.
- (vi) The changes from baseline in hsCRP, creatinine, nBNP, inflammatory cytokines (e.g. IL5, IL6, IL1β, IFNγ and TNFα) at 6 and 12 month visits, and mRNA expression markers of innate immunity at baseline and 12 month visits.
- (vii) The difference in the changes from baseline in diastolic and systolic blood pressure, heart rate and arterial stiffness at 6 and 12 months.
- (viii) The difference in the changes from baseline in total cholesterol, LDL-C, HDL-C, triglycerides, apo-B and apo-A at 12 months.

Tertiary outcomes

All tertiary assessments will involve intention-to-treat analyses among all randomised participants of daily dietary supplementation with vitamin D3 100µg vs placebo, vitamin D3 50µg vs placebo, and the difference between the two doses of vitamin D on:

- (i) The number of reported fractures at all sites, and at specific sites, during the 12 month treatment period.
 - (ii) The number of reported falls during the treatment period.
 - (iii) The change from baseline in muscle strength, and the differences between groups in physical performance and balance (assessed by the short physical performance battery) at 12 months.
 - (iv) Differences in heel-bone and wrist DEXA between treatment groups at 12 months.
 - (v) The change from baseline in vascular stiffness at 6 and 12 months.
 - (vi) The differences in short version Geriatric Depression Scale (GDS) scores
-

Chapter 6 : Effects of vitamin D on blood pressure and arterial stiffness

Results

Recruitment of study participants

Among the registered patients at the practice, 932 were invited to participate, 313 (33%) agreed and were offered a visit from the study nurse. Of these, 305 participants were randomised to take study treatment between 24 September 2012 and 14 March 2013 (**Figure 6-1**). Eight participants were not randomised (6 opted out and 2 were ineligible). All 6 month visits were completed by September 2013. The final study visit for the last participant was held on 10 March 2014. Among the surviving participants, 296 completed a 6-month visit, and 290 completed the final 12-month visit.

Baseline characteristics of randomised participants

Among the 305 participants, the mean (SD) age was 72 (6) years and 55% were aged 70 years or older (**Table 6-1**). About 51% were men overall with almost equal proportion of men in each of the treatment groups. About 20% had a prior history of CVD, 9% had diabetes and 39% had hypertension. About half of participants were prescribed blood pressure lowering drugs and about a quarter took statin therapy. Routine use of calcium was low and over-the-counter vitamin D supplements were taken by a tenth of the participants. The baseline characteristics of the participants were similar across the three treatment arms for age, baseline systolic and diastolic blood pressure and BMI. Background history of hypertension and the use of anti-hypertensive medications were more common among those taking 2000 IU daily.

Adherence within treatment and completeness of data at 6 months

The final follow-up visits were completed in 98% of the participants (**Table 6-2**) overall and in over 97% of participants within each of the treatment arms at 6 months (**Figure 6.2**). More than 85% of participants took their study treatment 'every day' or on 'most days' in the trial indicating good adherence.

Blood pressure data were available for 98% of all participants, but arterial stiffness data from the Arteriograph and Pulsetrace were only available in about 80% of the participants (due to technical and logistical reasons) and with broadly similar proportions of missing readings between treatment arms. Readings were available in 81%, 88% and 89% participants in the 4000 IU, 2000 IU and placebo groups, respectively. Pulsetrace PCA readings were available in 75%, 80% and 78% of participants taking 4000 IU, 2000 IU or placebo, respectively.

No adverse events for hypercalcaemia, hypercalciuria or renal stones were reported in any of the treatment groups. There were no significant differences in adverse events reported between groups, but all 3 deaths recorded in the trial were in the placebo group. More participants in the 4000 IU daily group reported infections but CVD events were comparable.

Plasma 25(OH)D levels and key variables at baseline

The mean (SD) baseline plasma 25(OH)D concentrations for all participants was 50.4 (17.5) nmol/L. Plasma concentrations of 25(OH)D were weakly correlated with physical activity

scores (self-reported physical activity on a scale of 1-10, 10 being for most active) and inversely correlated with weight and body mass index (**Table 6-3**) as may be expected with higher fat content. Baseline PWVao were weakly positively correlated with age, blood pressure (both systolic and diastolic) and with augmentation index, but not with the Pulsetrace measures (SI or RI) or with heart rate.

Effects of vitamin D treatment on plasma 25(OH)D concentrations

In the analysis of plasma 25(OH)D concentrations in the randomly selected 100 participants (**Figure 6-2**), 72% taking 4000 IU daily had a concentration >90 nmol/L, compared to 26% in those taking 2000 IU daily (9% among those taking placebo) after 1 month of treatment. After 6 months of taking treatment, mean (SD) 25(OH)D concentrations were 125 nmol/L, 100 (45) nmol/L, and 52.5 nmol/L among those allocated 4000 IU vitamin D3, 2000 IU vitamin D3, and placebo, respectively (**Table 6-4**). By the end of 12 months, mean plasma concentrations were 137 nmol/L and 102 nmol/L, respectively for those taking 4000 IU and 2000 IU daily compared to 53 nmol/L for those on placebo. This meant that while 88% of participants achieved >90 nmol/L among those who took 4000 IU daily, only 77% achieved these levels at the end of 12 months among those taking 2000 IU daily. There were no differences in adverse events, and there no events reported of significant hypercalcaemia, hypercalciuria or renal stones. The mean plasma concentrations of calcium and PTH were within normal ranges at the end of study (**Figure 6-2**).

After 12 months of treatment among those allocated 4000 IU, 2000 IU or placebo, 88%, 70% and 1%, respectively, achieved a 25(OH)D level >90 nmol/L at 12 months ($p<0.0001$

comparing 4000 IU daily versus placebo, comparing 2000 IU daily versus placebo, and for comparing 2000 IU daily versus 4000 IU). At 6 months, 86%, 64% and 2% respectively, achieved 25(OH)D >90nmol/L ($p<0.0001$ comparing 4000 IU daily versus placebo, comparing 2000 IU daily versus placebo, and $p<0.0003$ comparing 2000 IU daily versus 4000 IU). In addition, with 100 patients randomised in each group, the trial had >90% power at $2p=0.05$ and ~80% power at $2p=0.01$ to detect an increase in the proportion achieving a 12-month 25(OH)D concentration >90 nmol/L from 70% among those allocated 2000 IU daily to 90% among those allocated 4000 IU daily.)

Effects in pre-specified subgroups

The effects of 4000 IU daily versus 2000 IU daily of vitamin D on plasma levels of 25(OH)D after 12 months were similar in all the pre-specified sub-groups, except when grouped by body mass index (BMI). The absolute difference in achieved plasma 25(OH)D levels between the two active doses was significantly smaller among those with higher baseline BMI (**Figure 6-3**: p for trend <0.0001). The effects of 4000 IU versus 2000 IU dose of vitamin D on plasma levels of 25(OH)D were attenuated by one-third in those who were overweight and by two-thirds in those who were obese, compared to those with normal BMI. In a post-hoc analysis of the effects of 2000 IU vitamin D versus placebo alone, no differences in plasma 25(OH)D was found in any sub-group. The differences in plasma levels of 25(OH)D at 6 and 12 months between those allocated 4000 IU versus 2000 IU daily and versus placebo were broadly similar when assessed by quartiles of baseline plasma 25(OH)D levels (**Table 6-5**).

Effects of treatment on blood pressure and heart rate after 6 months

At the start of the study, the mean (SD) systolic blood pressure was 130.7 (19.2) mm Hg and the diastolic blood pressure was 76.5 (10.9) mm Hg in the randomised participants. While mean blood pressure was (**Table 6-6**) lower at 6 months compared to 12 months across all groups, there were no significant differences in final blood pressure or heart rate recorded at 6 and 12 months in any of the treatment groups. No differences in blood pressure or heart rate were identified when comparing those allocated vitamin D3 (either dose) or placebo, or between each of the allocated treatment arms.

Effects on arterial stiffness by Arteriograph measurement

Baseline mean (SD) PWVao was 9.7 (2)m/s and Aix was 35.4 (15) among participants in the placebo group. There were no significant differences in PWVao between those allocated to either of the vitamin D treatment groups or placebo after 6 months of treatment (**Table 6-6**), but Pulsewave velocity was found to be significantly higher in those taking 4000 IU daily compared to placebo. However, there were no differences in PWVao between those taking 2000 IU daily and those taking placebo, and no differences in effects between the two vitamin D treatment groups.

Effects on arterial stiffness by Pulsetrace PCA measurements

Baseline values for mean SI was 9.5 (3) and for RI was 66.9 (12) in the placebo group. There were no significant changes or consistent trends observed in both these indices (**Table 6-6**), either within each treatment arm, or between treatment groups after 12 months of taking vitamin D treatment.

Effects of vitamin D by sub-groups

No effects of taking either dose of vitamin D for 1 year was found on systolic blood pressure (**Table 6-7**) in any sub-group when analysed by tertiles of baseline plasma 25(OH)D concentrations, systolic blood pressure or age as recorded at randomisation. There were no effects on blood pressure for taking vitamin D supplements, among those reporting not using anti-hypertensive medications at randomisation. No effects were found when stratifying this group by tertiles of systolic blood pressure at randomisation either.

Among those with plasma 25(OH)D concentrations >55 nmol/L (top tertile), PWVao increased with vitamin D intake, but the differences between treatment groups did not reach statistical significance. When all participants were grouped by tertiles of age, those in the lowest tertile at randomisation had significantly higher PWVao at 12 months, if they were allocated to any vitamin D treatment (**Table 6-7**).

Among those not taking anti-hypertensive medications at randomisation, taking any vitamin D dose was associated with an increase in PWVao in all groups but these differences did not reach statistical significance. However, PWVao was significantly higher with vitamin D treatment among those in the lowest tertiles for age and systolic blood pressure at randomisation.

For Pulsetrace derived measures of arterial stiffness (RI and SI), no differences between treatment groups were identified, nor any consistent trends on the analysis by the above sub-groups.

Conclusions

In the BEST-D trial, where there was good adherence and tolerability of treatment, and dietary supplementation with vitamin D3 for 12 months significantly raised plasma 25(OH)D concentrations to greater than 90 nmol/L with no clinically evident adverse events or biochemical hazards associated with the intake. Moreover, supplementation with the two doses of vitamin D did not alter blood pressure or heart rate when compared to placebo, but appeared to increase pulsewave velocity albeit the effects did not reach statistical significance. Overall, the BEST-D study had limited power to detect significant effects of vitamin D on blood pressure and on arterial stiffness. Future work may involve pooling results from BEST-D with other similar trials of vitamin D to confirm or refute the results of BEST-D.

Chapter 6: Figures and Tables

Figure 6-1: Recruitment of participants in to the BEST-D trial

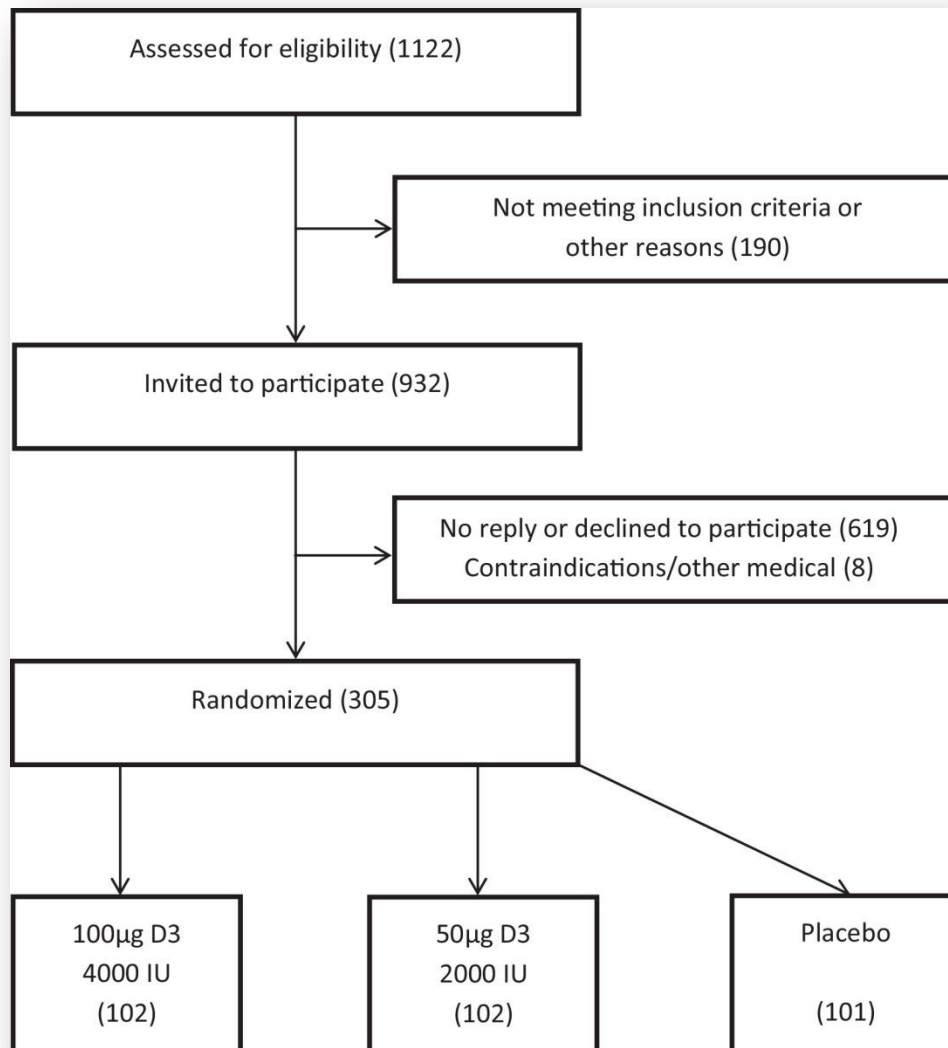


Figure 6-2: Effect of vitamin D allocation on mean plasma 25(OH)D, intact PTH and albumin-corrected calcium

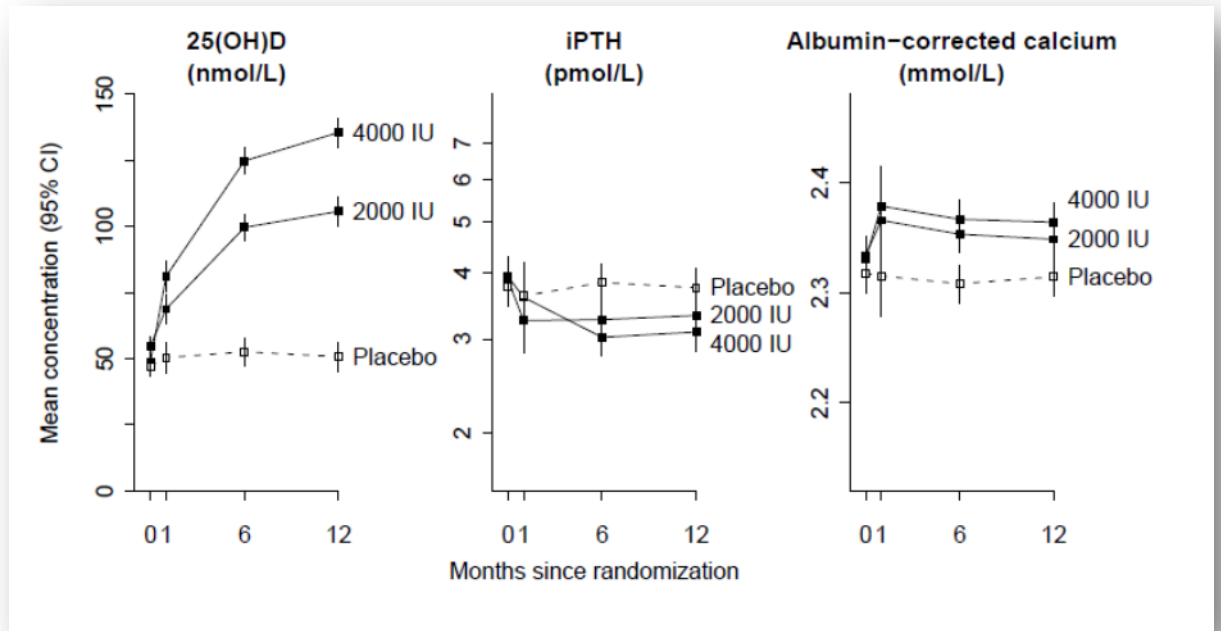


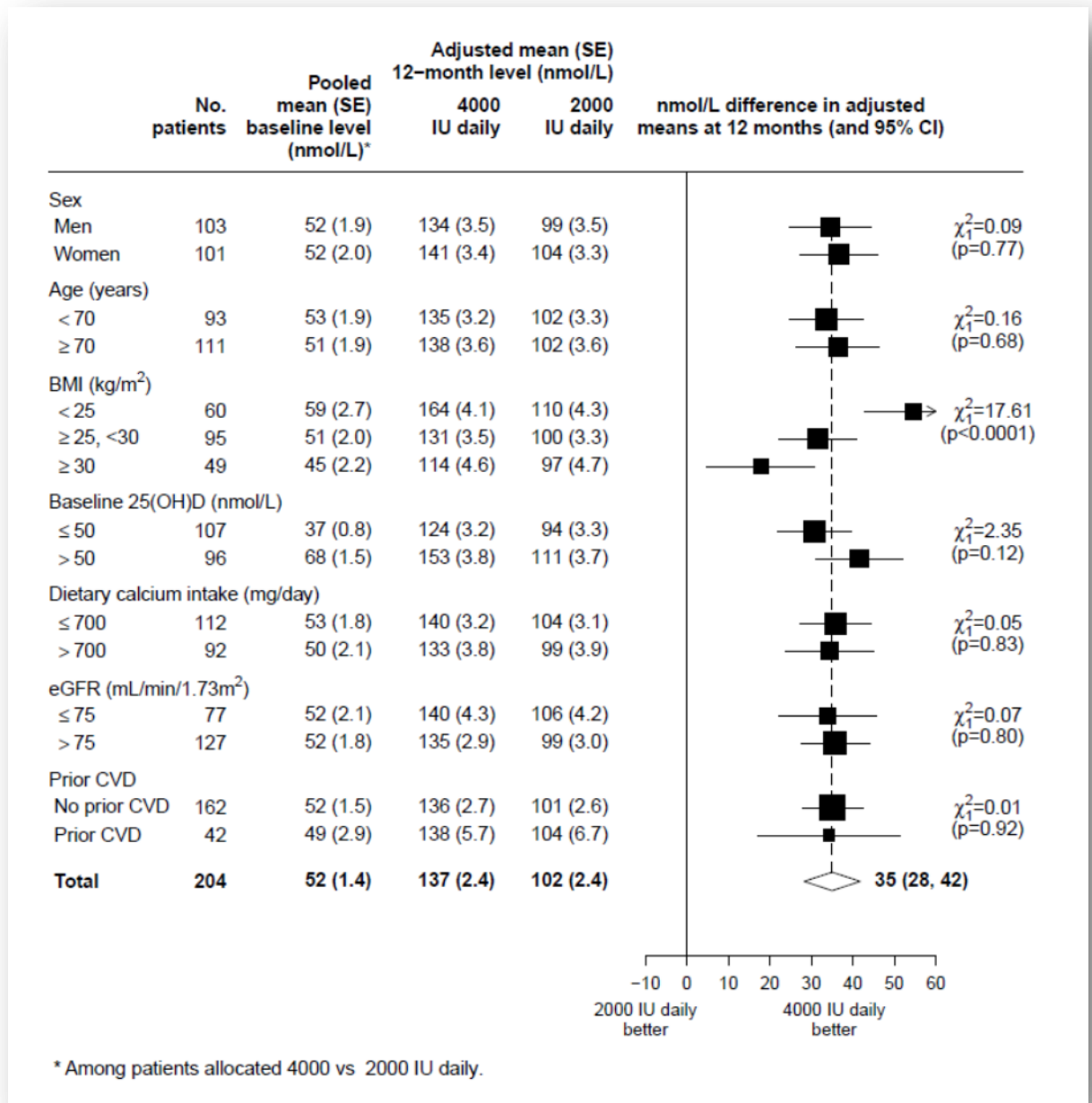
Figure 6-3: Effect of vitamin D allocation on mean plasma 25(OH)D levels by baseline sub-groups

Table 6-1: Baseline characteristics of participants in the BEST-D trial

| Mean (SD) or n, % | Allocated Treatment | | | |
|-----------------------------------|-----------------------|----------------------|---------------|---------------|
| | 100µg D3 (4000 IU) | 50µg D3 (2000 IU) | Placebo | Combined |
| Number randomised | 102 | 102 | 101 | 305 |
| Age - Mean (SD) | 71.8 (5.5) | 72.3 (6.1) | 72.1 (5.6) | 72.1 (5.7) |
| Male - n (%) | 52 (51%) | 52 (51.0%) | 52 (52%) | 156 (51%) |
| Current Smoker - n (%) | 7 (7%) | 7 (7%) | 7 (7%) | 21 (7%) |
| Prior disease | | | | |
| IHD* - n (%) | 20 (20%) | 11 (11%) | 11 (11%) | 42 (14%) |
| Stroke/TIA** - n (%) | 5 (5%) | 8 (8%) | 6 (6%) | 19 (6%) |
| Hypertension - n (%) | 40 (39%) | 44 (43%) | 35 (35%) | 119 (39%) |
| Diabetes - n (%) | 9 (9%) | 9 (9%) | 9 (9%) | 27 (9%) |
| Medication | | | | |
| Anti-hypertensive - n (%) | 44 (43%) | 50 (49%) | 40 (40%) | 134 (44%) |
| Statins - n (%) | 30 (29%) | 27 (27%) | 21 (21%) | 78 (26%) |
| Anti-platelets - n (%) | 18 (18%) | 19 (19%) | 15 (15%) | 52 (17%) |
| Vitamin D (<400 IU/d) - n (%) | 6 (6%) | 8 (8%) | 11 (11%) | 25 (8%) |
| Calcium - n (%) | 2 (2%) | 1 (1%) | 3 (3%) | 6 (2%) |
| Physical measurements | | | | |
| Height ,cm | 167 (10.0) | 168 (9.8) | 167 (10.1) | 167 (10.0) |
| Weight, Kg | 76.8 (17.1) | 77.7 (15.2) | 78.6 (14.9) | 77.7 (15.8) |
| BMI ,Kg/m ² | 27.2 (4.7) | 27.4 (4.1) | 28.0 (4.6) | 27.5 (4.5) |
| Systolic blood pressure, mmHg | 132 (22) | 132 (17) | 129 (18) | 131 (19) |
| Diastolic blood pressure, mmHg | 77 (11) | 77 (10) | 76 (11.5) | 77 (11) |

Table 6-2: Completed assessments, adherence and adverse events at 12 months of follow-up in the BEST-D trial

| | Arm 1 (4000 IU) N = 102 | Arm 2 (2000 IU) N= 102 | Placebo N = 101 |
|---|-------------------------------|------------------------------|--------------------|
| Number of completed follow-up visits at 12 months | 98 | 100 | 98 |
| Number of completed vascular measures | | | |
| Blood pressure, n | 98 | 100 | 98 |
| Missing data | | | |
| Arteriograph, % | 19 | 12 | 11 |
| Pulsetrace, % | 25 | 20 | 22 |
| Adherence[†], (%) | | | |
| Everyday | 64 | 74 | 69 |
| Most | 26 | 18 | 16 |
| Seldom/never | 5 | 5 | 9 |
| Unknown | 5 | 4 | 6 |
| Adverse Events | 29 | 30 | 25 |
| CVD | 3 | 2 | 2 |
| Infections | 10 | 5 | 6 |
| Deaths | 0 | 0 | 3 |

[†]Adherence was recorded as 'most' if <30 days treatment was missed, and as 'seldom or none' if <30 days treatment was taken. Counts are of the number of patients experiencing at least one event of the given type.

Table 6-3: Correlation between variables of interest in the BEST-D trial at baseline

| | Age | Plasma 25(OH)D | Height | Weight | BMI | SBP | DBP | HR | PWVao | Aix* | SI* | RI* | PPT | Physical activity |
|----------------|-------|-------------------|--------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----------------------|
| Age | 1 | -0.06 | -0.16 | -0.08 | 0.04 | 0.09 | -0.17 | -0.02 | 0.13 | 0.09 | -0.06 | -0.03 | 0.06 | -0.21 |
| 25(OH)D | -0.06 | 1 | 0.05 | -0.14 | -0.22 | -0.02 | 0.02 | -0.03 | -0.06 | 0.03 | 0.01 | 0.04 | -0.01 | 0.18 |
| Height | -0.16 | 0.05 | 1 | 0.60 | 0.00 | -0.05 | 0.15 | -0.12 | -0.05 | -0.37 | 0.35 | 0.21 | -0.08 | 0.02 |
| Weight | -0.08 | -0.14 | 0.60 | 1 | 0.80 | 0.08 | 0.24 | 0.01 | 0.05 | -0.31 | 0.26 | 0.15 | -0.11 | -0.26 |
| BMI | 0.04 | -0.22 | 0.00 | 0.80 | 1 | 0.13 | 0.18 | 0.10 | 0.09 | -0.14 | 0.06 | 0.03 | -0.08 | -0.34 |
| SBP | 0.09 | -0.02 | -0.05 | 0.08 | 0.13 | 1 | 0.78 | -0.08 | 0.21 | 0.43 | 0.16 | 0.17 | -0.13 | -0.06 |
| DBP | -0.17 | 0.02 | 0.15 | 0.24 | 0.18 | 0.78 | 1 | -0.02 | 0.20 | 0.32 | 0.26 | 0.25 | -0.20 | 0.04 |
| HR | -0.02 | -0.03 | -0.12 | 0.01 | 0.10 | -0.08 | -0.02 | 1 | 0.11 | -0.46 | 0.05 | -0.36 | -0.16 | 0.02 |
| PWVao | 0.13 | -0.06 | -0.05 | 0.05 | 0.09 | 0.21 | 0.20 | 0.11 | 1 | 0.28 | 0.01 | -0.08 | 0.01 | -0.05 |
| Aixao* | 0.09 | 0.03 | -0.37 | -0.31 | -0.14 | 0.43 | 0.32 | -0.46 | 0.28 | 1 | -0.13 | 0.07 | 0.06 | 0.04 |
| SI* | -0.06 | 0.01 | 0.35 | 0.26 | 0.06 | 0.16 | 0.26 | 0.05 | 0.01 | -0.13 | 1 | 0.67 | -0.86 | 0.02 |
| RI* | -0.03 | 0.04 | 0.21 | 0.15 | 0.03 | 0.17 | 0.25 | -0.36 | -0.08 | 0.07 | 0.67 | 1 | -0.62 | 0.03 |
| PPT | 0.06 | -0.01 | -0.08 | -0.11 | -0.08 | -0.13 | -0.20 | -0.16 | 0.01 | 0.06 | -0.86 | -0.62 | 1 | -0.01 |
| PA | -0.21 | 0.18 | 0.02 | -0.26 | -0.34 | -0.06 | 0.04 | 0.02 | -0.05 | 0.04 | 0.02 | 0.03 | -0.01 | 1 |

25(OH)D = plasma 25 hydrox-vitamin D3; SBP= Systolic blood pressure; DBP = diastolic blood pressure; BMI=body mass index; HR = heart rate; PWVao = Pulsewave velocity (aortic); Aixao = Augmentation index (aortic); SI = Stiffness index; RI = reflective index; PPT = Peak to peak time. * indicates indices. PA= Physical activity was a self-reported score based on a visual analog scale of 1-10, with '1' being inactive and '10' being very active.

Table 6-4: Effect of vitamin D on mean (SE) plasma levels of 25(OH)D, iPTH, albumin-corrected calcium and alkaline phosphatase

| | 4000 IU/day (n=102) | 2000 IU/day (n=102) | Placebo (n=101) | P¹ | P² | P³ |
|--|--------------------------------|--------------------------------|----------------------------|----------------------|----------------------|----------------------|
| Plasma 25(OH)D, nmol/L | | | | | | |
| Baseline | 49 (1.5) | 55 (2.2) | 47 (1.5) | - | - | - |
| 1 month | 81 (1.9) | 69 (1.8) | 49 (1.9) | <0.0001 | <0.0001 | <0.0001 |
| 6 months | 126 (2.4) | 97 (2.4) | 55 (2.4) | <0.0001 | <0.0001 | <0.0001 |
| 12 months | 137 (2.4) | 102 (2.4) | 53 (2.4) | <0.0001 | <0.0001 | <0.0001 |
| Plasma iPTH, pmol/L | | | | | | |
| Baseline | 3.93 (0.193) | 3.86 (0.148) | 3.76 (0.157) | - | - | - |
| 6 months | 2.98 (0.070) | 3.25 (0.076) | 3.91 (0.093) | <0.0001 | <0.0001 | 0.0092 |
| 12 months | 3.05 (0.080) | 3.31 (0.087) | 3.82 (0.100) | <0.0001 | 0.0001 | 0.0283 |
| Plasma albumin-corrected calcium, mmol/L | | | | | | |
| Baseline | 2.33 (0.012) | 2.33 (0.008) | 2.32 (0.007) | - | - | - |
| 6 months | 2.36 (0.006) | 2.35 (0.006) | 2.32 (0.006) | <0.0001 | <0.0001 | 0.05 |
| 12 months | 2.36 (0.006) | 2.34 (0.006) | 2.32 (0.006) | <0.0001 | 0.0106 | 0.0386 |
| Plasma alkaline phosphatase, IU/L | | | | | | |
| Baseline | 58 (1.7) | 61 (1.6) | 59 (1.9) | - | - | - |
| 6 months | 57 (0.7) | 58 (0.7) | 59 (0.7) | 0.0247 | 0.13 | 0.46 |
| 12 months | 60 (0.9) | 60 (1.0) | 60 (1.0) | 0.99 | 1.00 | 0.98 |

Arithmetic mean (SE) shown for 25(OH)D and albumin-corrected calcium and geometric mean (approximate SE) shown for iPTH and alkaline phosphatase. Means at 1, 6 and 12 months are adjusted for the baseline values, with missing data imputed using multiple imputation. Mean iPTH and albumin-corrected calcium levels were not pre-specified outcomes.

¹ P-value comparing 4000 IU daily versus placebo.

² P-value comparing 2000 IU daily versus placebo.

³ P-value comparing 4000 versus 2000 IU daily.

Table 6-5: Effect of vitamin D on plasma 25(OH)D concentrations at 6 and 12 months, by baseline quartiles of 25(OH)D

| Baseline 25(OH)D, nmol/L | 6 months | | | 12 months | | |
|-----------------------------|------------------|------------------|------------|------------------|------------------|------------|
| | 2000 IU daily | 4000 IU daily | Difference | 2000 IU daily | 4000 IU daily | Difference |
| <36 | 89 (4.6) | 115 (5.0) | 26 (6.8) | 91 (4.5) | 123 (5.0) | 32 (6.7) |
| 36 to <48 | 93 (4.8) | 112 (4.0) | 20 (6.3) | 98 (4.6) | 120 (3.9) | 22 (6.1) |
| 48 to <60 | 92 (5.9) | 132 (5.0) | 40 (7.7) | 103 (5.9) | 141 (5.1) | 38 (7.8) |
| 60+ | 111 (4.0) | 145 (4.9) | 34 (6.3) | 116 (4.6) | 167 (5.6) | 50 (7.3) |

Adjusted mean (SE) shown. Adjusted for baseline values with missing data imputed using multiple imputation. Four baseline groups defined by the quartiles of the distribution in all participants.

Table 6-6: The effects of allocation to vitamin D supplementation on plasma 25(OH)D, blood pressure, heart rate and arterial stiffness after 6 and 12 months of follow-up

| | 4000 IU/day (n=102) | 2000 IU/day (n=102) | Placebo (n=101) | P [§] | P [†] | P [§] |
|------------------------------------|------------------------|------------------------|--------------------|----------------|----------------|----------------|
| ARTERIOGRAPH MEASUREMENTS | | | | | | |
| Systolic blood pressure (mm Hg) | | | | | | |
| 6 months | 129.7 (1.31) | 129.9 (1.36) | 127.8 (1.44) | 0.34 | 0.29 | 0.90 |
| 12 months | 132.5 (1.43) | 131.8 (1.51) | 131.8 (1.51) | 0.71 | 0.98 | 0.73 |
| Diastolic blood pressure (mm Hg) | | | | | | |
| 6 months | 75.9 (0.91) | 76.5 (0.95) | 75.5 (1.02) | 0.81 | 0.49 | 0.63 |
| 12 months | 77.2 (0.89) | 77.0 (0.94) | 76.6 (0.96) | 0.65 | 0.73 | 0.92 |
| Heart rate (beats/min) | | | | | | |
| 6 months | 64.9 (0.81) | 65.3 (0.85) | 66.7 (0.92) | 0.14 | 0.27 | 0.71 |
| 12 months | 66.0 (0.84) | 66.4 (0.87) | 67.0 (0.87) | 0.40 | 0.64 | 0.72 |
| Pulse wave velocity (m/s) | | | | | | |
| 6 months | 9.8 (0.13) | 9.9 (0.13) | 9.9 (0.13) | 0.46 | 0.89 | 0.55 |
| 12 months | 10.1 (0.13) | 9.9 (0.14) | 9.6 (0.14) | 0.0088 | 0.20 | 0.19 |
| PULSE TRACE MEASUREMENTS | | | | | | |
| Aortic augmentation index (%) | | | | | | |
| 6 months | 36.7 (1.34) | 37.5 (1.38) | 35.0 (1.33) | 0.36 | 0.19 | 0.68 |
| 12 months | 37.5 (1.28) | 36.8 (1.36) | 37.1 (1.38) | 0.83 | 0.86 | 0.69 |
| Pulse trace stiffness index (m/s) | | | | | | |
| 6 months | 9.6 (0.25) | 9.6 (0.24) | 9.5 (0.24) | 0.70 | 0.85 | 0.83 |
| 12 months | 9.4 (0.28) | 9.4 (0.27) | 9.5 (0.36) | 0.94 | 0.98 | 0.96 |
| Pulse trace reflection index (m/s) | | | | | | |
| 6 months | 64.6 (1.40) | 65.4 (1.31) | 65.2 (1.34) | 0.75 | 0.89 | 0.66 |
| 12 months | 66.3 (2.17) | 68.4 (2.53) | 66.3 (2.32) | 0.98 | 0.54 | 0.52 |

Mean (SE) shown

Estimates adjusted for baseline values with missing data imputed using multiple imputation

§ P-value comparing 4000 IU daily versus placebo.

† P-value comparing 2000 IU daily versus placebo.

\$ P-value comparing 4000 versus 2000 IU daily.

Table 6-7: Effects of vitamin D3 on blood pressure and arterial stiffness by baseline characteristics

| | Either dose (n=204) | Placebo (n=101) | P |
|---|------------------------|--------------------|--------|
| SYSTOLIC BLOOD PRESSURE, mm Hg (ARTERIOGRAPH) | | | |
| By tertiles of 25(OH)D at randomisation | | | |
| <=40 | 135.4 (1.90) | 131.8 (2.41) | 0.24 |
| >40, <=55 | 130.1 (1.64) | 133.1 (2.19) | 0.28 |
| >55 | 131.3 (1.98) | 130.0 (2.97) | 0.71 |
| By tertiles of 25(OH)D at randomisation (among those not on antihypertensive drugs at randomisation) | | | |
| <=40 | 134.6 (2.44) | 130.5 (3.51) | 0.34 |
| >40, <=55 | 129.6 (2.59) | 133.6 (2.95) | 0.31 |
| >55 | 130.8 (2.64) | 131.0 (4.29) | 0.96 |
| By tertiles of SBP at randomisation | | | |
| <=121 | 118.2 (1.68) | 118.3 (2.50) | 0.99 |
| >121, <=137 | 131.5 (1.61) | 132.8 (2.07) | 0.62 |
| >137 | 147.0 (2.10) | 143.7 (3.09) | 0.38 |
| By tertiles of age at randomisation | | | |
| <=68 | 130.4 (1.62) | 131.0 (2.26) | 0.82 |
| >68, <=73 | 132.8 (1.86) | 131.2 (2.52) | 0.60 |
| >73 | 133.9 (2.16) | 133.7 (3.12) | 0.97 |
| PULSE WAVE VELOCITY, m/s (ARTERIOGRAPH) | | | |
| By tertiles of 25(OH)D at randomisation | | | |
| <=40 | 10.0 (0.16) | 9.6 (0.21) | 0.11 |
| >40, <=55 | 9.9 (0.18) | 9.8 (0.24) | 0.63 |
| >55 | 9.9 (0.18) | 9.3 (0.27) | 0.07 |
| By tertiles of 25(OH)D at randomisation (among those not on antihypertensive drugs at randomisation) | | | |
| <=40 | 10.1 (0.22) | 9.4 (0.31) | 0.08 |
| >40, <=55 | 10.0 (0.27) | 9.7 (0.29) | 0.37 |
| >55 | 9.9 (0.24) | 9.1 (0.37) | 0.10 |
| By tertiles of SBP at randomisation | | | |
| <=121 | 9.8 (0.17) | 9.3 (0.25) | 0.05 |
| >121, <=137 | 10.1 (0.15) | 9.7 (0.21) | 0.12 |
| >137 | 10.0 (0.18) | 9.8 (0.26) | 0.64 |
| By tertiles of age at randomisation | | | |
| <=68 | 9.9 (0.15) | 9.2 (0.20) | 0.0145 |
| >68, <=73 | 9.9 (0.18) | 9.4 (0.25) | 0.11 |
| >73 | 10.2 (0.19) | 10.3 (0.27) | 0.81 |
| PULSE TRACE STIFFNESS INDEX, m/s | | | |
| By tertiles of 25(OH)D at randomisation | | | |
| <=40 | 8.5 (0.35) | 8.7 (0.46) | 0.76 |
| >40, <=55 | 9.9 (0.35) | 9.9 (0.56) | 0.92 |
| >55 | 9.9 (0.34) | 9.8 (0.67) | 0.91 |
| By tertiles of 25(OH)D at randomisation (among those not on antihypertensive drugs at randomisation) | | | |
| <=40 | 9.2 (0.52) | 9.1 (0.71) | 0.87 |
| >40, <=55 | 9.9 (0.48) | 9.9 (0.65) | 0.99 |
| >55 | 9.8 (0.48) | 10.4 (0.95) | 0.60 |

Effects of vitamin D3 on blood pressure and arterial stiffness by baseline characteristics

(cont...)

| | Either dose (n=204) | Placebo (n=101) | P |
|-------------------------------------|------------------------|--------------------|------|
| By tertiles of SBP at randomisation | | | |
| ≤121 | 9.0 (0.31) | 9.8 (0.48) | 0.18 |
| >121, ≤137 | 9.6 (0.36) | 9.2 (0.51) | 0.57 |
| >137 | 9.8 (0.37) | 9.4 (0.62) | 0.63 |
| By tertiles of age at randomisation | | | |
| ≤68 | 9.9 (0.32) | 9.7 (0.48) | 0.80 |
| >68, ≤73 | 9.5 (0.34) | 9.9 (0.54) | 0.59 |
| >73 | 8.7 (0.37) | 8.7 (0.72) | 0.97 |

Mean and SE estimates are adjusted for the baseline values, with missing data imputed using multiple imputation

Chapter 7: Analyses and Conclusion

Introduction

In this thesis, I explored the role of low vitamin D in the aetiology of CVD, by reviewing the observational studies and clinical trials of vitamin D and CVD outcomes, including evaluating causality of these associations. In the Whitehall prospective study, baseline plasma 25(OH)D concentrations and their relationships with cause-specific mortality was examined and found inverse log-linear associations. The meta-analyses of prospective studies included the findings from Whitehall, where the 'optimal levels' of plasma 25(OH)D concentrations for the lowest risks of CVD was observed to be around 80-90 nmol/L. The thesis reported on the outline and design of the BEST-D trial, a dose-finding study that compared the effects of two higher doses of vitamin D (2000 IU and 4000 IU daily) on biochemical outcomes in older people for one year, and helped identify the optimum dose of vitamin D supplement to test in a future large trial evaluating effects on CVD and other disease outcomes. There were no statistically significant effects of supplementation with vitamin D3 on blood pressure (a cardiovascular risk factor) and on arterial stiffness (a surrogate marker of CVD) in the trial of older people (BEST-D) after 1 year.

Key aims of the thesis

The key aims of this thesis were to review the evidence from prospective studies of vitamin D and CVD, and the effects of supplementation with vitamin D in

randomised-controlled trials on CVD outcomes. Another aim was to describe the outline and design of a dose-finding trial of vitamin D to determine the optimum dose of vitamin D3 to be used in a future trial evaluating effects on fracture, CVD and other disease outcomes. The final aim was to report the effects of 2 daily doses of vitamin D3 (2000 IU and 4000 IU daily), on blood pressure (CVD risk marker) and arterial stiffness (surrogate marker for CVD) when compared to placebo.

Low vitamin D status and CVD

The review of prospective studies of vitamin D demonstrated that low vitamin D was widely prevalent and optimal levels of plasma 25(OH)D for CVD outcomes was unknown. Most studies reported inverse associations between measured 25(OH)D concentrations at baseline and CVD outcomes including mortality, but many studies had few participants, and had inconsistent methodology of reporting and analysis of results. Hence there was still uncertainty whether these reported associations could be due to the effects of reverse causality or due to residual confounding. The shape and size of these associations were unclear and the plasma concentrations associated with the lowest risks for CVD mortality were not identified. Further, it was also possible that plasma 25(OH)D concentration was a marker of frailty and that these associations were simply coincidental and not causal.

Whitehall study: Key findings

Inverse associations of plasma 25(OH)D with CVD and non-CVD causes of mortality

The Whitehall resurvey of 5409 older British men included 3215 deaths and was one of the largest and longest prospective studies at the time that examined the associations of baseline 25(OH)D with cause-specific mortality, and showed an approximately log-linear inverse association of plasma 25(OH)D concentrations with both CVD and non-CVD causes of mortality within the range of 30 to 90 nmol/L (Chapter 3). After adjustment for age, season, prior disease, markers of health and frailty, and other cardiovascular risk factors, a 2-fold higher plasma concentration of 25(OH)D was associated with one fifth lower risk of CVD mortality (20% lower CVD mortality; 23% lower risks for non-vascular mortality). The change in the Chi-squared statistic after adjustment for established risk factors would suggest that it is possible that these associations may reflect incomplete adjustment for established risk factors that were measured imprecisely, or for unknown risk factors that may not have been measured. However, the shape and strength of these associations were broadly similar among men with and without prior disease (defined as those with vascular disease, cancer or diabetes) as reported at baseline and the associations persisted after excluding deaths during the first five years of follow-up, another method for minimising the effects of reverse causation, and is similar to those that other studies detected.^{59, 197}

It is possible that low vitamin D status could reflect frailty and that low plasma concentrations of 25(OH)D may simply reflect a sicker population with one or more co-morbidities with associated higher risks of death. As plasma 25(OH)D concentrations are lower in those who have low physical activity and in those who are obese, the associations with CVD may be due to confounding by these covariates. However, in the Whitehall study, excluding the deaths after 5 years of blood collection did not attenuate these associations, nor did the adjustment for self-reported health or frailty. In another prospective analysis by Dobnig et al, in those with low to average physical activity, low plasma 25(OH)D concentrations were associated with higher risks of CVD deaths.^{197, 275}

Meta-analyses of prospective studies of vitamin D

We compared the findings from the Whitehall resurvey, which included only older men living in the UK, to those from other prospective studies that reported on CVD and all-cause mortality to evaluate consistency. The meta-analyses of prospective studies of 25(OH)D included about 4600 CVD deaths, and over 11,700 deaths from all causes. These meta-analyses confirmed a consistent trend in the inverse associations of plasma 25(OH)D concentrations with CVD mortality and with all-cause mortality. Individuals in the top vs. bottom quarter of 25(OH)D concentrations had 21% (95% CI: 13–28%) lower CVD mortality and 28% (95% CI: 24–32%) lower all-cause mortality. Although there was a significant trend in the effect size reported by study when studies were ordered by size, consistent with publication bias (where smaller studies tend to report more extreme findings),

other meta-analyses including one which examined individual participant-level data found similar inverse associations between baseline 25(OH)D and both CVD and all-cause mortality.²⁷⁵ At the time, this report was one of the first meta-analyses of plasma 25(OH)D concentrations and all-cause and cardiovascular mortality.²⁷⁶

When the published findings from studies that enrolled only women were compared with those that recruited men alone, the relative risks of all-cause mortality were greater in women with lower plasma 25(OH)D concentrations compared to those on the top quartile (Chapter 3). Women tend to have lower mean plasma 25(OH)D concentrations attributed mainly to their relatively higher body fat content, and hence the inverse associations of 25(OH)D with disease may be more pronounced in women. There is less clarity as to whether the low plasma 25(OH)D concentrations from being female or obese, indicate increased risks of mortality, as optimal concentrations could be lower in such groups; but this will need testing in a future trial.²⁷⁷

Optimal levels of plasma 25(OH)D concentration for CVD

From the Whitehall resurvey and the meta-analysis of prospective studies, the lowest risks for CVD mortality were observed at plasma 25(OH)D concentrations between 80-90 nmol/L, which appear to indicate the optimal plasma concentrations for CVD and be the potential target plasma concentrations to achieve in a future larger trial of vitamin D supplements for prevention of CVD and other disease outcomes. These are broadly consistent with other analyses

published since,^{275, 278} and in line with recommendations for optimal plasma 25(OH)D concentrations (>75 nmol/L) for other health outcomes.⁵³ Some prospective studies have reported a possible J-shaped or curvilinear association of baseline 25(OH)D concentrations with all-cause and CVD mortality^{107, 143, 275} especially when plasma 25(OH)D concentrations were higher than 120 nmol/L.^{140, 141} The number of participants with such high concentrations in most meta-analyses were small,^{141, 275, 279} and these findings warrant further testing in large clinical trials. We were unable to confirm or refute these findings in the Whitehall study, again due to the limited number of participants with plasma 25(OH)D concentrations greater than 100-120 nmol/L.

Lack of specificity of associations of 25(OH)D

In the Whitehall study, given age, a doubling in 25(OH)D concentration (on the log-scale) was associated with almost a third lower risk of CVD mortality and with a similar lower risk for non-vascular mortality. The relative risks for CVD and all-cause mortality between top versus bottom quartiles reported in other meta-analyses were generally higher than those reported in this study,^{142, 275, 278} but the similarities in the shape and strength of the associations of plasma 25(OH)D concentrations with both CVD and non-CVD causes of death would argue against a causal relationship of low vitamin D with CVD by conventional statistics..

Vitamin D may influence CVD through a number of mechanisms but also through some fundamental mechanism that may be common to different disease conditions

(Chapter 2 and **Figure 2-2**). For example, the effects of vitamin D in modulating the immune response could influence the pathophysiology of a diverse range of conditions including CVD, cancer and infection.⁷ Vitamin D can affect multiple health outcomes, as VDRE in genes influences 10% of all the genes and there is evidence of local metabolism of the vitamin in multiple cell types especially those involved with inflammation and immunity. Alternatively, the effects of vitamin D on CVD may be mediated through mechanisms that are independent of known cardiovascular risk factors, and hitherto unidentified. Hence, the lack of specificity could be consistent with the biological actions of the vitamin and may not necessarily exclude causality.

Limitations of the Whitehall study and meta-analyses

Among the limitations of the Whitehall study, data on prior disease, health status and physical activity at re-survey were self-reported. Hence, while the strength of the associations was attenuated substantially after adjustment for these measures, it is possible that residual confounding by poor health status may have persisted despite the efforts to minimise the effects of it. Formal assessment of physical activity was not made at baseline, but participant-reported self-rated health and ability to undertake activities of daily living (based on a 15 point questionnaire) was used to adjust analyses. Self-reported physical activity is unreliable compared to more objective measures;²⁸⁰ other objective methods could have had an impact on the strengths of these associations. No data were collected on the precise time of day at which blood samples were collected in this study and, hence, it was not

possible to assess the effects of diurnal variation in plasma levels of 25(OH)D on disease outcomes.

Any non-response bias (by preferentially excluding frail older people) should have minimized rather than accentuated the effects of reverse causality (**Table 3-1**). As causes of deaths were supplied by the Office of National Statistics some misclassification of the causes of death in this population could have occurred, which would dilute any real differences between the different causes of death.²⁸¹ However, most large prospective studies of vitamin D utilised a similar methodology for ascertaining the causes of death.

Most men in the Whitehall analysis had a single baseline assessment of their plasma 25(OH)D concentration, but the correlation between repeated measurements of log plasma 25(OH)D concentration recorded in 187 men over a 1.5 year period was 0.64. Other prospective studies have also found similar correlations for repeated measures of 25(OH)D concentrations, suggesting single measures of 25(OH)D can be utilised in prospective studies.^{282, 283} Since individuals were classified on the basis of a single measurement of plasma 25(OH)D, it is possible that the true strength of the mortality associations observed with long-term average or usual 25(OH)D concentrations may be substantially steeper. Hence, the mortality associations with long-term usual concentrations of 25(OH)D may be 50% more extreme.²⁴⁰

The prospective studies of vitamin D included in the meta-analyses used differing assays for measuring 25(OH)D concentrations and hence the real risk reduction with doubling of plasma 25(OH)D, may differ to the risk reduction estimates described in our study. Other meta-analyses as discussed above, found greater risk reductions with increasing plasma 25(OH)D concentrations, and this may mean that the inverse associations between low vitamin D and CVD may be even greater.²⁷⁵

Finally, a rigorous methodology for the epidemiological analysis was used in the Whitehall study and meta-analysis and although a number of strategies to minimise the effects of confounding and of reverse causality (within the limits of the study design) were utilised, prospective studies such as the Whitehall resurvey, are unable to provide conclusive proof on the causal relevance of plasma 25(OH)D concentrations in the development of CVD.

Generalisability of Whitehall study findings

Most men in this study were living in and around Greater London area at the time of blood collection. Although not representative of the wider UK population, in contrast to other large prospective studies, our study design would have reduced the likelihood of confounding by geographic factors and variations in sunshine exposure. The Whitehall resurvey only included older men, but when the findings were compared to those from other prospective studies (**Table 4-2**), the strength and shape of these associations were found to be broadly consistent with other studies which included participants from the general population, and with the

findings published in other subsequent meta-analyses. Only a handful of prospective studies were unable to find similar associations between 25(OH)D concentrations and all-cause mortality (Chapter 2, **Table 2-1**) but generally only when participants had higher mean 25(OH)D concentrations with a narrow range of distribution, or if they were recruited after acute events such as a heart attack.²⁰¹

Review of the randomised trials of vitamin D

Need for good quality evidence on CVD outcomes

Review of previously published trials of vitamin D showed that these trials were not designed to evaluate effects of vitamin D on CVD outcomes as the trials did not use sufficiently high enough doses of the vitamin D supplement to achieve or maintain ‘optimal levels’ of plasma 25(OH)D. The previous trials were primarily designed to evaluate effects on non-CVD outcomes such as falls and fractures. Meta-analyses of vitamin D trials on CVD outcomes did not confirm benefits for taking vitamin D, but much of the data in these meta-analyses came from the WHI trial of post-menopausal women, being the largest vitamin D trial to date (more than 36,000 post-menopausal women), which showed no effects on CVD or blood pressure. As discussed in Chapter 2, the null results from this trial will influence any findings and inferences^{185, 284} made about the effects of vitamin D on CVD outcomes, until such time newer trials are able to provide further data.

The effects of supplementation with vitamin D on blood pressure lowering remains controversial, with conflicting results being reported from the clinical trials of

vitamin D (which showed no effects on blood pressure lowering overall) and the Mendelian randomisation studies (that report a predisposition for lower blood pressure in those with a higher vitamin D status). From the few trials that were available, there were no effects of vitamin D on other CVD risk markers such as CRP, inflammation, or lipids, or the effects remain unclear (Chapter 2).^{167, 221} Whether vitamin D intake could modify arterial stiffness measures was not known, as only two small trials (**Table 2-2**), one in young African American teenagers²²² and the other in 100 patients with type 2DM,²¹⁹ reported a reduction in aortic stiffness measures.

Vitamin D intake and mortality

Several meta-analyses of randomised trials of vitamin D report a modest mortality benefit among those taking vitamin D supplements. However, there is a lack of clarity as to the type, frequency and dose of supplementation of vitamin D that is associated with such benefits. One analysis reported mortality reduction with calcium and vitamin D supplementation, but not for taking vitamin D alone,¹⁹¹ while another found benefits with vitamin D3 intake and not vitamin D2.¹⁴² Intermittent high doses of vitamin D intake was associated with an increased incidence of falls and fractures,¹⁰⁰ and vitamin D3 was more efficacious in achieving target plasma 25(OH)D concentrations compared to vitamin D2.⁹³ There was very little data on the safety and efficacy of taking daily high doses of vitamin D3 for long periods,¹⁵ especially in the older people.

The magnitude of possible benefits for reduction in all-cause mortality when taking vitamin D was unclear. The doses used in the previous trials were insufficient to achieve optimal plasma 25(OH)D concentrations, and yet the meta-analyses of these trials demonstrated a 1% to 7% relative risk reduction in mortality in those taking vitamin D.^{188, 190} Based on the Whitehall resurvey and the observational studies,⁹ even greater reduction in all-cause mortality may be possible if optimal plasma concentrations of 25(OH)D were to be achieved by the use of higher dose of vitamin D supplements. What is clear is that there is a need for a larger trial of vitamin D using a high enough dose to evaluate the effects on CVD outcomes.

The dose of vitamin D supplement to be used

Before a large trial of vitamin D to evaluate effects on CVD outcomes is to be undertaken, the daily dose of vitamin D3 that could achieve optimal concentrations of 25(OH)D in most people needed to be established. Review of the observational studies and meta-analyses, and our own analysis (Chapters 2-4) identified the lowest risks for CVD at plasma 25(OH)D concentrations of 80-90 nmol/L. Concentrations of 25(OH)D >75 nmol/L were associated with the lowest risks for other adverse health outcomes such as falls, fractures and cancer.^{11, 53} In the UK, as mean 25(OH)D levels among people over 65 years of age range between 32-55 nmol/L, vitamin D3 supplements >2000 IU daily would be necessary to achieve optimal 25(OH)D concentrations. Data on safety and efficacy of such high daily doses of vitamin D3 were limited, especially for use in the UK.

The BEST-D trial – Key findings

Optimal plasma 25(OH)D concentrations were achieved

BEST-D (Biochemical Efficacy and Safety Trial of vitamin D) was the first randomized placebo-controlled trial to assess the effects of two higher oral doses of vitamin D₃, 4000 IU and 2000 IU (equivalent to 100 µg and 50 µg) daily, in older adults living in the UK for 1 year. The trial demonstrated a higher proportion of participants taking 4000 IU daily maintained plasma concentrations >90 nmol/L at the end of 1 year compared to those taking 2000 IU or placebo, and the intake of such doses were not associated with clinically apparent adverse effects (Chapters 5 and 6). The dose-response was curvilinear with a plateau in the achieved plasma 25(OH)D concentrations occurring after 4 weeks of vitamin D intake. This trial addressed a key gap in our knowledge on dose response of daily supplementation with high dose vitamin D₃.

This work adds to the literature on vitamin D dosing trials and provided information on the safety and efficacy of using higher doses in the older Caucasian people, living in the UK. In a recent dose-finding trial of 163 post-menopausal women, serial doses of increasing vitamin D between 400 IU to 4800 IU daily were given, and the peak doses corresponded to plasma concentrations of about 120 nmol/L after 1 year, and were consistent with the results from BEST-D.⁹¹ However, hypercalcaemia and hypercalciuria defined by biochemical methods were detected in this trial which monitored urine and blood calcium every 3 months, but these events did not correlate with the dose of vitamin D administered, and no patients reported

significant adverse events. We found no reported adverse effects in the BEST-D participants, and found no significant hypercalcaemia at the end of 12 months. There are no other trials that I am aware of, which used doses between 2000 IU to 3000 IU daily of vitamin D3 and reported significant adverse effects associated with such intake. The results from BEST-D now confirm that intake of up to 4000 IU of vitamin D3 in the older people are well tolerated and safe over 1 year.

Effects on blood pressure and arterial stiffness with vitamin D intake

Among BEST-D participants, taking 2000 IU or 4000 IU vitamin D3 daily for 1 year had no effects on systolic or diastolic blood pressure, heart rate, or on the measures of arterial stiffness compared to placebo. Despite significant differences in achieved plasma 25(OH)D concentrations, with an almost doubling of concentrations from baseline in the 4000 IU arm, no significant effects were detected on the CVD risk markers after 1 year either. In sub-group post-hoc analyses, taking either dose of vitamin D was associated with an increase in the Pulsewave velocity (measured by Arteriograph), and this was more apparent in those who were younger and in those with lower systolic blood pressure at enrolment. Other measures of arterial stiffness remained unchanged. These effects on arterial stiffness were not statistically significant after taking account of multiple testing.

The lack of effects for vitamin D on blood pressure in the treatment arms or in any of the sub-groups of gender, baseline vitamin D status or blood pressure, is

consistent with the findings of a recent meta-analysis of trials of vitamin D and blood pressure,¹⁸³ which found no effects on any pre-specified sub-groups. The lack of statistically significant effects for taking vitamin D on arterial stiffness measures was broadly consistent with other trials, especially those that have included older Caucasian participants (**Table 2-2**).^{175, 221} No other trial of vitamin D had used such higher daily doses of vitamin D (2000 IU and 4000 IU) and evaluated effects on arterial stiffness markers in the elderly.

Explaining the lack of effects of vitamin D on CVD risk factors in the BEST-D trial

Though concentrations of plasma 25(OH)D >90 nmol/L were achieved in the BEST-D study, and mean concentrations were in the optimal range for CVD, no beneficial effects of supplementation on CVD risk factors were observed with vitamin D intake. This is contrary to the observational data of vitamin D, but it may mean that the optimal concentrations need to be maintained for longer if any beneficial effects were to become apparent. Autier et al, noted mortality reduction benefits associated with vitamin D intake would need 6 years to become apparent.¹⁸⁸

Perhaps, the trial participants in BEST-D were predominantly healthy and hence no further beneficial effects of vitamin D intake on risk factors could have been possible. For instance, the blood pressure lowering effects may not have been apparent in this relatively normotensive population. Alternatively, there could have been insufficient power to detect small effects of vitamin D on blood pressure lowering and arterial stiffness (discussed below). In BEST-D, there was >90% power

(at $2p = 0.05$) to detect a difference in BP of 10 mm Hg between those taking any vitamin D versus placebo. If the effects of vitamin D on blood pressure and arterial stiffness were smaller than anticipated, this would have been difficult to detect in this trial.

Could the higher plasma concentrations achieved with vitamin D treatment (>100 nmol/L in both groups) be associated with the lack of effects on CVD? In the Whitehall study, the log-linear inverse associations between plasma 25(OH)D and CVD mortality was observed in the range between 30-90 nmol/L; at higher concentrations the associations appeared to become attenuated (**Figure 3-2**). If higher plasma concentrations of 25(OH)D are associated with increased CVD risks as reported in some prospective studies, achieving 'supra-normal' concentrations could have attenuated any beneficial effects of supplementation with vitamin D. The mean concentrations of plasma 25(OH)D achieved in the BEST-D trial were higher than those previous trials that showed blood pressure lowering with vitamin D supplements.¹⁷² However, the post-hoc analyses of the BEST-D participants, found no blood pressure lowering effects even in sub-groups, when participants were grouped by baseline age, systolic blood pressure, or after excluding those who took anti-hypertensive treatment.

Aspects of the BEST-D trial assessments of CVD risk markers may explain the null results, as the vascular measurements were conducted at participant homes rather than in a laboratory setting. Although, these methods for estimating arterial stiffness (Arteriograph and PulseTrace PCA II) were relatively new and had little

validation for CVD outcomes, there was good correlation of repeated measures at 6 months within the placebo group participants, and the standard error of the measurements were also small indicating the measures were robust. We also aimed to reduce the effects of diurnal variation and that of temperature fluctuations on blood pressure and arterial stiffness in a pragmatic manner, by ensuring follow-up appointments were scheduled at the same time of the day wherever possible. These assessments at participant homes could have introduced measurement variability, but mean (SE) values of the Arteriograph and PulseTrace measures of arterial stiffness were broadly comparable to those from other published studies.

Limitations of the BEST-D trial

The BEST-D trial was designed predominantly to assess the efficacy, safety and tolerability of two higher doses of vitamin D3 supplements for use in a larger trial of vitamin D in future. However, as the study recruited older participants living at a single geographic location and registered under a single GP Practice, generalisability of the trial findings and of the effects of vitamin D on CVD to the wider UK population, which include younger people and those from other ethnic backgrounds, is uncertain. The dose of vitamin D to achieve optimal plasma 25(OH)D concentrations in non-Caucasian populations may be different, as African-American women appear to require >2000 IU daily to achieve optimal concentrations.^{285 286} No data were collected on the precise time of day at which blood samples were collected in this study and, hence, it was not possible to assess the effects of diurnal variation in plasma levels of 25(OH)D on disease outcomes.

However, appointments were scheduled at the same time of day in serial visits to minimise diurnal variation in blood pressure and arterial stiffness, which also minimise the effects of diurnal variation in the effects of vitamin D on plasma levels of 25(OH)D.

Although the main trial was designed to confirm biochemical effects, the trial design was suitable as a preliminary, explorative, pilot analysis to evaluate the effects of vitamin D on CVD risk markers. However, the design of the BEST-D trial may have obscured potential effects of vitamin D on blood pressure and arterial stiffness. The recruited participants were from a healthy cohort of patients, who were motivated, educated and interested in participation in the trial. Although the adherence was good with treatment and the follow-up visit completion being exemplary, this is also indicative of a highly motivated participant group, and who are likely to be healthier. Hence demonstrating CVD risk reduction may have been difficult.

The minimisation algorithms used for the randomised allocation of double-blinded treatment did not include blood pressure, arterial stiffness or baseline 25(OH)D concentrations, and could have introduced inequities in baseline characteristics that could have attenuated the effects of taking vitamin D. However, further, attempts to refine allocation with these above factors could have posed considerable logistical problems and it would not have been feasible to implement such complex allocation in a trial of this size. However, we conducted post-hoc analysis to further examine the effects of vitamin D supplements by sub-groups of baseline blood

pressure, arterial stiffness, plasma 25(OH)D concentrations, and use of anti-hypertensive medications, and have interpreted these findings with caution, as such post-hoc analysis can introduce bias.

Trials generally lack sufficient power to detect effects of vitamin D on CVD

The lack of effects of vitamin D on CVD measures in BEST-D could reflect the inadequate sample size of the treatment groups, as only a >10% change in systolic blood pressure or pulsewave velocity would have been detectable. The BEST-D trial had >90% power to detect a 10% change in pulsewave velocity (about 1m/s) or a 10% reduction in systolic blood pressure between any dose of vitamin D intake and placebo, but would not have identified smaller changes (power to detect <5% differences in pulsewave velocity was < 50% and in blood pressure was <70%). Despite this, our trial had more participants than most other vitamin D trials evaluating the effects on blood pressure, included in the recent meta-analysis.¹⁸³ However, as the Mendelian randomisation studies demonstrate a blood pressure reduction of only 2-3 mm Hg (with 10 nmol/L difference in plasma 25(OH)D concentration and in a cohort of more than 45,000 participants), the previous trials of vitamin D including BEST-D maybe significantly under-powered to detect such smaller magnitude of effects on blood pressure. However, achieving higher plasma concentrations of 25(OH)D with the use of higher doses of vitamin D could translate into a greater reduction in blood pressure (with time), but this is yet to be proven in a randomised controlled trial.

Higher concentrations of plasma 25(OH)D and the risks of CVD need testing

We could not find evidence that higher plasma concentrations of 25(OH)D was associated with higher risks of CVD and/or all-cause mortality in the Whitehall study (Chapter 3, **Figure 3-2**), but there were only few participants with 25(OH)D concentrations >120 nmol/L in this study. The curvilinear or J-shaped association with all-cause mortality at 25(OH)D concentrations > 120 nmol/L described in other studies may be because sicker individuals may take more vitamin D supplements raising plasma 25(OH)D concentrations, but have shorter life spans.

In the BEST-D trial, those taking any dose of vitamin D3 daily had higher arterial stiffness after 1 year, with mean plasma concentrations of 25(OH)D reaching above 100 nmol/L in both vitamin D treatment groups (and 120 nmol/L in the 4000 IU treatment group). Although these effects did not reach statistical significance, it is possible that the effects on arterial stiffness could be due to increased calcification of the vasculature. The increased arterial stiffness was more apparent among the young participants and in those participants with the lowest systolic blood pressure. This could mean that the effects of vitamin D were harder to detect in the other participants as they had stiffer vessels (due to advanced age or higher blood pressure). Furthermore, there was a dose-dependent rise in serum calcium levels in the BEST-D participants, but the serum calcium levels remained in normal range with no hypercalcaemia detected. The trial was not powered to detect differences in secondary outcomes and hence these findings need to be interpreted with caution.

Higher risks for falls and fractures with high intermittent doses of vitamin D are reported in trials and may be explained by the increased physical ability due to sufficient vitamin D status leading to higher chance of falls and fractures. However, it may also be due to the differences in handling of high dose vitamin D supplements by the body. For instance, vitamin D3 supplementation was associated with reduced mortality but not the intake of vitamin D2. Vitamin D production from sun exposure is self-regulating, and the continued exposure to heat and light causes breakdown of 7-dehydroxy-cholesterol. Perhaps, the lack of such temperature and light sensitive mechanisms to regulate the extraneous supply of vitamin D may cause altered calcium handling, and in the absence of clinically apparent hypercalcaemia.

However, it would seem unlikely that higher plasma 25(OH)D concentrations could cause harm as plasma levels of 25(OH)D of 115 nmol/L are reported among the Masai and Hadzabe tribes living in equatorial East Africa, people thought to live with very little changes to their hunter-gatherer life-styles for centuries.^{252, 287} Further, concentrations of 25(OH)D >120 nmol/L are achievable after prolonged sunlight exposure, and hence these concentrations are within physiologic ranges. What is not clear is whether such high levels among the Caucasian populations are necessary, or if they could have adapted to the lower UVB availability.⁴⁶ In the Whitehall analyses, the range associated with the lowest risks of mortality was around 75-90 nmol/L and is consistent with analysis including participant data from the US and Northern Europe.^{9, 275} The observational associations may not necessarily indicate higher risks with supplementation of vitamin D, but no trials to

date have tested the effects of maintaining plasma 25(OH)D concentrations at such levels for long periods on CVD outcomes. However, the possibility that higher concentrations of plasma 25(OH)D could be linked with higher mortality risks, cannot be excluded and needs testing.

Low 25(OH)D is not an indicator of frailty

Studies that reported inverse relationships between plasma 25(OH)D and CVD outcomes have not been able to exclude the possibility that plasma 25(OH)D concentrations could indicate frailty or of inactivity. In the Whitehall study, correction for self-reported physical activity did not eliminate the observed risks. In the LURIC study, Dobnig et al found that in those who had average or below-average activity levels, lower 25(OH)D was associated with higher risks of dying, when participants grouped by 'below average', 'average' and 'above average' self-reported physical activity.¹⁹⁷ However, the effects of lower risks of all-cause mortality with vitamin D supplements demonstrated in the trials of vitamin D could mean that vitamin D is a modifiable risk factor, and not an indicator of ill-health. Due to the lack of large adequately designed trials of vitamin D, there is simply insufficient data to conclude otherwise.

Implications for further research

The findings of the inverse associations of plasma 25(OH)D concentrations with higher risks of CVD mortality and with all-cause mortality are of considerable public

health interest, as low plasma 25(OH)D concentrations are common in the population and can be modified by vitamin D supplements.

The discrepancy between the randomised trials of vitamin D showing lack of effect for taking vitamin D on CVD outcomes and mortality, and the Mendelian randomisation studies showing possible benefits of optimal vitamin D status, needs further evaluation.^{193, 194} Could the lack of effects on CVD reported from the vitamin D randomised trials be due to, the use of inadequate dose and duration of vitamin D supplements, the concomitant use of calcium supplements, the form and frequency of supplementation, or an inadequate sample size of the trials? Although a number of trials are already underway using higher vitamin D doses and evaluating CVD and other disease outcomes, (Chapter 2, **Table 2-4**) these individual trials may still not be able to find meaningful differences in mortality or other CVD outcomes and a meta-analysis of such trials may be needed.

However, the sample size required to demonstrate a statistically significant effect may be even greater than all the participants in the on-going trials put together. A further large randomised trial may be the best approach to test and confirm the potential causal relevance of vitamin D for CVD. Even if the effects of supplementation with vitamin D on disease outcomes are modest, use of supplements to modify plasma 25(OH)D concentrations are effective and could be a cost-effective way of reducing risks of death from CVD and other causes, in the

general population. Importantly, it may afford a new therapeutic target for the both primary and secondary prevention of CVD.

Whether a higher plasma concentrations greater than 120 nmol/L are associated with harm is not yet proven and needs testing. In the BEST-D trial, any intake of vitamin D appeared to increase arterial stiffness when compared to placebo, but these results were not statistically significant after taking account of multiple testing.

In the UK, there is a need to test and establish the relevance of supplementation with vitamin D in relation to CVD and other health outcomes. The recommendations for vitamin D requirements for health may indeed be influenced by geographic location, ethnic background or physical factors, and may be different for different populations. People in the UK are at a particular disadvantage as they are unable to produce vitamin D after sun exposure during the winter months, and it is unclear whether these insufficiencies have a cumulative, additive and detrimental effect on health or it is simply the effects of an adaptation for living in areas of low UVB availability.

Significance of the thesis findings

The findings presented in this thesis provide valuable new information on the associations between vitamin D and CVD. The Whitehall study was one of the first reports to demonstrate the inverse associations between vitamin D and CVD

mortality, identify and suggest an optimal plasma 25(OH)D concentrations for CVD outcomes and the reported meta-analyses which confirmed the shape and strength of the associations between vitamin D and CVD was one of the first of such studies to evaluate the association from other prospective studies. However, given the limitations of a prospective analysis, this study was unable to confirm whether this is a causal association.

The BEST-D trial is one of the largest dose-finding trials of vitamin D₃, and the first to test the safety and efficacy of using two daily doses of vitamin D₃ (4000 IU and 2000 IU daily) among the older people living in the UK. The trial showed that both doses were likely to achieve and maintain plasma 25(OH)D concentrations >90 nmol/L throughout the year, although proportionally more participants had plasma concentrations >90 nmol/L in the group that received 4000 IU daily. Neither dose was associated with clinically detectable adverse effects or biochemically significant hypercalcaemia. This information will help design a larger trial evaluating the effects of vitamin D supplements on CVD outcomes in future.

The trial demonstrated no significant effects of taking higher doses of vitamin D₃ on blood pressure (CVD risk factor) or arterial stiffness. However, the trial was not powered to detect small differences in either of these measures, and a possible benefit or hazard for taking vitamin D supplements over the longer term cannot be confirmed or refuted based on the BEST-D trial findings.

Recommendations for future research

There is substantial public health interest in addressing the question of causality of low vitamin D and CVD, and based on the evidence presented in this thesis, this need to be proven. An appropriately designed randomised controlled trial of vitamin D supplements evaluating effects on risk of fracture, CVD and other disease outcomes as primary outcomes is necessary. The appropriateness of modifying mean population 25(OH)D levels through fortification or supplementation, as a preventative public health measure needs further evidence.

Such a trial will require a large number of participants to detect possibly modest but potentially worthwhile effects on disease outcomes. The design of such a trial has substantial logistic and cost implications and is challenging to obtain support. Perhaps innovative trial designs could be utilised, including embedding the trial in a previously recruited cohort study, such as the UK Biobank or GP register could simplify recruitment and follow-up of trial participants and reduce costs. So far the on-going trials of vitamin D will only recruit about 50,000 to 60,000 participants, but even larger trials may be required for a more robust analysis of benefit or harm on CVD and other health outcomes.

Conclusions

Plasma 25(OH)D concentrations were inversely associated with CVD mortality. Previous trials were unable to confirm whether these associations were causal. The BEST-D study has shown that two daily doses of vitamin D3 (2000 IU and 4000 IU)

are broadly safe and efficacious in raising plasma 25(OH)D concentrations in older people living in the UK. The trial also demonstrated that vitamin D had no effects on CVD risk markers after 1 year of intake. A large well-designed randomised controlled trial of vitamin D supplements evaluating effects on CVD and other disease outcomes such as fractures and cancer as primary outcomes is necessary, and such a trial will require a large number of participants and a long duration of treatment to detect effects on major disease outcomes.

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Appendix

- 1. European Heart Journal: Whitehall and meta-analyses paper**
- 2. Plasma 25(OH)D assay for Whitehall**
- 3. BEST-D protocol paper**
- 4. Data Analysis plan for BEST-D**
- 5. Ethics Approval for BEST-D**
- 6. Whitehall study ethics approval**



Vitamin D and risk of death from vascular and non-vascular causes in the Whitehall study and meta-analyses of 12 000 deaths

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Received 6 July 2012; revised 18 October 2012; accepted 16 November 2012

Aims

To examine the independent relevance of plasma concentrations of 25-hydroxyvitamin D [25(OH)D] for vascular and non-vascular mortality.

Methods and results

We examined associations of plasma concentrations of 25(OH)D and cause-specific mortality in a prospective study of older men living in the UK and included findings in meta-analyses of similar studies identified by a systematic search reporting on vascular and all-cause mortality. In a 13-year follow-up of 5409 men (mean baseline age 77 years), 1358 died from vascular and 1857 from non-vascular causes. Median season-adjusted baseline 25(OH)D concentration was 56 (interquartile range: 45–67) nmol/L. After adjustment for age and seasonality, higher concentrations of 25(OH)D were inversely and approximately linearly (log–log scale) associated with vascular and non-vascular mortality throughout the range 40–90 nmol/L. After additional adjustment for prior disease and cardiovascular risk factors, a doubling in 25(OH)D concentration was associated with 20% [95% confidence interval (CI): 9–30%] lower vascular and 23% (95% CI: 14–31%) lower non-vascular mortality. In meta-analyses of prospective studies, individuals in the top vs. bottom quarter of 25(OH)D concentrations had 21% (95% CI: 13–28%) lower vascular and 28% (95% CI: 24–32%) lower all-cause mortality.

Conclusions

Despite strong inverse and apparently independent associations of 25(OH)D with vascular and non-vascular mortality, causality remains uncertain. Large-scale randomized trials, using high doses of vitamin D, are required to assess the clinical relevance of these associations.

Keywords

Vitamin D • Cardiovascular disease • Mortality

Introduction

Prospective observational studies have reported that low circulating concentrations of 25-hydroxyvitamin D [25(OH)D] are associated with higher risks of cardiovascular disease,^{1–7} cancer,^{6,8–10} and all-cause mortality.^{5,7,11–13} However, as low 25(OH)D concentrations are correlated with several known vascular risk factors, it is possible that any such associations with disease may reflect confounding by these risk factors. Alternatively, these associations with 25(OH)D may be due to reverse causation, as individuals with vascular disease or cancer, who may be frail or unwell,

may be more likely to stay indoors, and have low plasma 25(OH)D concentrations due to inadequate sunlight exposure. Previous meta-analyses of prospective studies reported a significant inverse association of 25(OH)D with all-cause mortality,^{14,15} but did not distinguish vascular from non-vascular causes of death.

The relevance of measurements of circulating 25(OH)D levels in the general population, including those with vascular disease, is uncertain. No large randomized trials of vitamin D have yet been completed with vascular disease or cancer as the primary outcome. Previous meta-analyses of randomized trials of vitamin D reported only borderline statistically significant effects of

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vitamin D treatment on all-cause mortality,^{16,17} but were unable to detect significant effects on vascular outcomes.¹⁸ These trials typically used daily doses of 400–800 IU of vitamin D₃, which may not be sufficient to optimize plasma 25(OH)D concentrations throughout the year.¹⁹ If the inverse associations of 25(OH)D with vascular disease and other outcomes are causal and reversible by treatment, then this could have important implications for public health, particularly for countries in the Northern hemisphere²⁰ where low vitamin D levels are highly prevalent.

We examined the associations of plasma concentrations of 25(OH)D with vascular and non-vascular causes of mortality in a 13-year follow-up of a prospective study of 5409 older men living in the UK in 1997, and compared the results in meta-analyses of similar prospective studies of 25(OH)D and vascular and all-cause mortality. The aims of the present study were: (i) to examine cross-sectional associations of 25(OH)D with other vascular risk factors; (ii) to assess the shape and strength of the associations of plasma 25(OH)D concentrations with vascular and non-vascular causes of death, overall and separately in those with and without any pre-existing disease; and (iii) to compare these results in meta-analyses of prospective studies of 25(OH)D and vascular and all-cause mortality.

Methods

Study population

The Whitehall study is a prospective study of 19 019 male civil servants who were working in London at the time of recruitment in 1967–70.^{21,22} Following a successful pilot study in 1995, a resurvey was conducted of all surviving 8448 participants in this cohort during 1997–98, after approval by the relevant ethics committees of the participating institutions. Data were collected for the resurvey using mailed questionnaires seeking details of previous medical history (diagnoses of heart attack, angina, stroke, cancer, and diabetes), self-reported health status, medications taken in the past month, lifestyle characteristics (e.g. smoking status and alcohol consumption), and last known civil service employment grade. All 7044 (83%) respondents were subsequently sent a blood collection kit and asked to attend their local surgery to have a sample collected and measurements of blood pressure, height, and weight recorded. Complete data on questionnaires and relevant blood results were available from 5409 (77% of respondents) men for the present report. Compared with responders, non-responders were older and had a lower previous employment grade, but response rates were not associated with total cholesterol levels recorded from 1967 to 70.²³

Laboratory methods

Whole-blood samples (~10 mL) were returned by mail in sealed tubes (Vacutainer; Becton Dickson, Franklin Lakes, NJ, USA) containing potassium ethylenediaminetetraacetic acid with 0.34 mmol/L of aprotinin. These samples were mailed at room temperature to the CTSU Wolfson Laboratory in Oxford, with 78% arriving within 24 h and 96% arriving within 48 h of blood collection [mean time in post: 1.3 days (range 0–7 days)]. On arrival in the laboratory, the blood was centrifuged, and plasma was aliquoted and stored at –40°C. Plasma 25(OH)D concentrations were measured using an automated immunoassay on the IDS-iSYS analyser (Immunodiagnostic systems, Boldon, UK). In addition, 187 of these men had plasma 25(OH)D

concentrations measured on blood samples collected for the pilot study for this resurvey, on average 1.5 years prior to the main resurvey. The IDS-iSYS 25(OH)D assay standardization is traceable to ultraviolet quantification and calibration was verified by comparison with an isotope dilution liquid chromatography-tandem mass spectrometry method. Over the testing period, proficiency testing with vitamin D external quality assessment scheme samples indicated that the IDS-iSYS assay was systematically overestimating concentrations compared with the mean obtained for all 25(OH)D assays. Hence, a linear correction, multiplying by 0.757, was applied to all measured 25(OH)D concentrations. Plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, apolipoprotein A₁ (Apo A₁), and apolipoprotein B (Apo B) as well as biomarkers of inflammation [C-reactive protein (CRP), fibrinogen, and albumin] and cystatin C were measured using standard methods.²² Estimated glomerular filtration rate (eGFR, in mL/min/1.73 m²) was calculated from cystatin C concentration using the formula $eGFR = 80.35/\text{cystatin C (in mg/L)} - 4.32$.²⁴

Cause-specific mortality follow-up

Participants were flagged for mortality at the Office for National Statistics (UK), which provided the date and cause [including International Classification of Disease (ICD) codes] of all deaths occurring until end of August 2010. The mean follow-up period among survivors was 13.1 years. Cause-specific mortality was coded using ICD-9 up to August 2002 and ICD-10 subsequently. Vascular deaths (heart disease, stroke, and other vascular disease) were defined if coded as ICD-9 codes 390–459, 798 or ICD-10 codes I00–I99, R96, with all other causes of death being defined as non-vascular. Deaths from ischaemic heart disease (IHD: ICD-9 codes 410–414; ICD-10 codes I20–I25), stroke (ICD-9 codes 430–438; ICD-10 codes I60–I69), cancer (ICD-9 codes 140–208; ICD-10 codes C0–C97), and respiratory disease (ICD-9 codes 460–519; ICD-10 codes J00–J99) were also analysed separately.

Statistical methods

Plasma 25(OH)D concentrations were analysed on the logarithmic scale. Since concentrations of plasma 25(OH)D varied throughout the year, the values were adjusted for month of blood sampling. This was done by adding to each participant's log 25(OH)D measurement the difference between the overall mean log 25(OH)D concentration (seen in all men) and the mean observed just among men sampled in the same month. Since the majority of men were examined in the summer of 1997, when 25(OH)D concentrations tended to be highest (see Supplementary material online, Figure S2), this adjustment tended to shift values towards those seen in summer rather than winter months. Men were subsequently classified into five equal sized groups on the basis of their season-adjusted 25(OH)D concentration, and the means and prevalences of other baseline characteristics, adjusted for age, were estimated [with tests for linear trend between log 25(OH)D and the risk factor performed using, respectively, linear or logistic regression adjusted for age]. To assess the shape of the association between 25(OH)D and mortality, men in the top and bottom fifths were further divided in half for the purpose of the plotting of dose–response relationships to better characterize any relationships at more extreme concentrations. Relative risks (RRs) (estimated by hazard ratios in Cox models) were estimated for each group relative to the lowest and are shown as 'floating absolute risks' [which does not alter their values but merely ascribes a 95% confidence interval (CI) to the RR in every group].²⁵ The average RR corresponding to a doubling in 25(OH)D concentration (approximately a

2 SD increase on the log scale) was also estimated. The proportionality assumption of the Cox model was assessed using the method described by Grambsch and Therneau.²⁶ Analyses were done before and after adjustment for age, prior history of disease (myocardial infarction, angina, stroke, cancer, or diabetes), self-reported health status (on a four point scale), ability to perform particular activities of daily living (based on a 15-point questionnaire), smoking status (current smoker, ex-smoker, and never smoker), alcohol consumption, last known employment grade, blood pressure (both at entry to Whitehall study in 1967–70 and at resurvey in 1997–98, as well as treatment for hypertension at resurvey), body mass index, blood lipids, and apolipoproteins (LDL-C, HDL-C, Apo A₁, and Apo B), markers of inflammation (CRP, albumin, and fibrinogen), and eGFR. To further assess the effects of reverse causality, analyses were repeated separately in men with and without a prior history of disease (defined as above), while further analyses excluded deaths during the first 5 years of follow-up.

Meta-analyses of prospective studies

Data from the Whitehall study were included in meta-analyses of all published reports of prospective studies that reported associations of circulating concentrations of 25(OH)D with either vascular mortality or all-cause mortality before January 2012. Eligible population-based studies (see Supplementary material online, Table S1), based on prespecified selection criteria, were identified by electronic literature searches (PubMed, Embase, and Cochrane databases) using a systematic search strategy (see Supplementary material online, Figure S1). Studies were to be included if they (i) involved a prospective (or nested case-control) study design; (ii) included more than 200 adult participants recruited from the general population; and (iii) had data on 25(OH)D concentrations and risk of death from cardiovascular or all-causes. Studies were excluded if they were selected on the basis of (i) diagnosis of prior disease (cancer, vascular, or renal disease); (ii) risk factors (diabetes and hypertension); (iii) nursing home residents; (iv) participants in clinical trials; or (v) meta-analyses of previous studies. Data were abstracted from each report on RRs (commonly hazard ratios from Cox regression models) and their 95% CI for vascular and all-cause mortality, and verified by two authors working independently. For each study, the most fully adjusted RR and its 95% CI were extracted. Any RR models that had been adjusted for calcium and phosphate were not included (as these were considered to be on the causal pathway). Where necessary, the RRs were recalibrated to correspond to the top vs. bottom quarter of the 25(OH)D distribution (most common approach taken in individual studies).²⁷ This was done by estimating the number of SDs that each published RR would have corresponded to [on some normal transformation of the underlying 25(OH)D distribution] before recalibrating the log RR (and its standard error) to correspond to a 2.54 SD difference (since 2.54 is the difference in mean values between the top and bottom quarters of a normal distribution). Principal investigators were contacted and asked to provide additional data on the SD of 25(OH)D concentration to facilitate standardized comparisons. Overall summary estimates of the effect were calculated using the Mantel–Haenszel inverse-variance weighted method for meta-analysis. In forest plots, studies were ordered according to the amount of statistical information they contributed to the overall result [and, for display only, were grouped as being: ‘small’ (providing <1% of the total information provided by all the studies); ‘medium’ (1 to <10%); or ‘large’ (at least 10%)]. All *P* values were two sided and *P* values <0.05 were deemed

conventionally significant. Analyses were done using SAS version 9.1 (SAS Institute, Cary, NC, USA) and R version 2.11.1 (www.r-project.org).

Results

Baseline characteristics

Selected characteristics of the 5409 men included in the analyses are summarized in Table 1. The mean age of participants at resurvey was 76.9 (SD 4.9) years, and about one-third (1841 men) had a history of prior vascular disease, cancer, or diabetes at resurvey. The majority (87%) were non-smokers, while 78% were self-reported ‘current’ alcohol drinkers.

Distribution of 25-hydroxyvitamin D concentrations at baseline

Plasma concentrations of 25(OH)D varied substantially by month of blood collection, and, even after adjustment for month of blood collection, concentrations had a log-normal distribution (see Supplementary material online, Figure S2). Median 25(OH)D concentration (standardized for month of blood collection) was 56 nmol/L (interquartile range 45–67 nmol/L) (Table 1). In a sample of 187 men with repeated measurements taken 1.5 years apart, the self-correlation in log 25(OH)D was 0.64. At any given age, men with higher 25(OH)D concentrations were less likely to have a history of vascular disease, cancer, or diabetes, and less likely to have been diagnosed with hypertension or taking treatment for hypertension, than men with lower concentrations. Measured systolic blood pressure at resurvey in 1997 was only weakly related with 25(OH)D concentrations, and blood pressure at the initial examination for the Whitehall study in 1967–70 was unrelated with 25(OH)D concentrations. Men with higher 25(OH)D also had lower mean body mass index than men with lower 25(OH)D and were less likely to have been of manual/clerical grade at retirement. In contrast, men with higher plasma 25(OH)D concentrations had higher mean LDL-C, HDL-C, ApoA₁, and albumin concentrations, and lower mean CRP and fibrinogen concentrations, than men with lower 25(OH)D concentrations.

Association of 25-hydroxyvitamin D with vascular and non-vascular mortality

Overall among the 5409 participants, 3215 men died during over 50 000 person years of follow-up (overall death rate: 6.4% per year; mean follow-up among survivors 13 years), including 1358 deaths (2.7% per year) from vascular causes and 1857 deaths (3.7% per year) from non-vascular causes (Table 2). Among the 3568 men without a history of vascular disease, cancer, or diabetes, there were 727 deaths (2.0% per year) from vascular causes and 1124 deaths (3.1% per year) from non-vascular causes. After classifying men into seven groups based on season-adjusted 25(OH)D concentration, higher concentrations of 25(OH)D were inversely and, on the log–log scale, approximately linearly related to the risk of vascular and, at least throughout the range 40–90 nmol/L, of non-vascular mortality in

Table 1 Study characteristics, overall and by prior disease, and baseline 25-hydroxyvitamin D concentration

| Baseline characteristics | All men | No prior disease | Prior disease | Fifth of baseline 25(OH)D ^a | | | | | p ^{†b} |
|---|--------------|------------------|---------------|--|--------------|--------------|--------------|--------------|-----------------|
| | | | | I | II | III | IV | V | |
| Number of men | 5409 | 3568 | 1841 | 1081 | 1082 | 1082 | 1082 | 1082 | |
| Age (years) | 76.9 (4.9) | 76.5 (4.8) | 77.6 (5.0) | 78.9 (5.2) | 77.1 (5.1) | 76.7 (4.6) | 76.1 (4.5) | 75.5 (4.3) | |
| 25(OH)D (nmol/L) | 56 (45–67) | 57 (47–68) | 54 (43–65) | 36 (5) | 48 (3) | 56 (2) | 65 (3) | 83 (18) | |
| Medical history (%) | | | | | | | | | |
| IHD | 19.9 | 0.0 | 58.4 | 22.0 | 20.5 | 20.3 | 19.8 | 16.5 | 0.002 |
| Stroke | 7.2 | 0.0 | 21.1 | 9.3 | 6.6 | 6.7 | 6.7 | 5.9 | 0.001 |
| CVD | 24.9 | 0.0 | 73.3 | 28.1 | 25.4 | 25.4 | 24.6 | 20.7 | <0.001 |
| Diabetes | 5.9 | 0.0 | 17.4 | 8.8 | 6.7 | 5.8 | 2.8 | 4.0 | <0.001 |
| Cancer (not skin) | 7.9 | 0.0 | 23.2 | 10.1 | 8.1 | 7.4 | 6.8 | 6.5 | 0.006 |
| Self-reported health good/excellent | 77.4 | 85.3 | 62.0 | 67.1 | 75.8 | 79.9 | 80.5 | 85.5 | <0.001 |
| Manual/clerical socio-economic grade at baseline | 17.4 | 17.7 | 16.8 | 20.2 | 19.4 | 16.2 | 15.8 | 14.9 | <0.001 |
| Lifestyle (%) | | | | | | | | | |
| Current tobacco smoker | 12.7 | 14.1 | 10.2 | 16.8 | 12.3 | 12.7 | 10.1 | 11.4 | <0.001 |
| Current alcohol drinker | 77.9 | 79.2 | 75.4 | 73.3 | 75.8 | 80.5 | 79.5 | 80.8 | <0.001 |
| Blood pressure and body mass | | | | | | | | | |
| Diagnosis of hypertension/use of blood | 42.0 | 33.1 | 59.3 | 45.0 | 43.1 | 41.4 | 40.2 | 40.5 | 0.003 |
| Systolic blood pressure (mm Hg) | 144.8 (20.1) | 145.4 (19.8) | 143.7 (20.7) | 144.0 (20.5) | 144.6 (20.1) | 145.2 (20.1) | 144.9 (20.2) | 145.5 (20.3) | 0.05 |
| Diastolic blood pressure (mm Hg) | 80.2 (10.8) | 80.9 (10.7) | 78.8 (10.9) | 79.6 (11.0) | 80.4 (10.8) | 80.1 (10.8) | 80.1 (10.8) | 80.5 (10.8) | 0.10 |
| Body mass index (kg/m ²) | 25.2 (3.2) | 25.1 (3.2) | 25.4 (3.3) | 25.5 (3.3) | 25.4 (3.2) | 25.4 (3.2) | 25.1 (3.2) | 24.8 (3.2) | <0.001 |
| Blood pressure measured in 1967–70 (~30 years earlier; mm Hg) | | | | | | | | | |
| Systolic blood pressure | 130.8 (17.6) | 129.5 (17.2) | 133.3 (18.2) | 130.5 (17.9) | 130.9 (17.5) | 131.3 (17.5) | 130.5 (17.6) | 130.5 (17.7) | 0.57 |
| Diastolic blood pressure | 80.3 (12.1) | 79.4 (11.7) | 82.0 (12.6) | 80.1 (12.3) | 80.6 (12.1) | 80.8 (12.1) | 80.1 (12.1) | 79.9 (12.2) | 0.35 |
| Laboratory measurements | | | | | | | | | |
| LDL-C (mmol/L) | 3.37 (0.79) | 3.40 (0.78) | 3.31 (0.80) | 3.28 (0.79) | 3.36 (0.78) | 3.42 (0.78) | 3.42 (0.78) | 3.36 (0.78) | <0.001 |
| HDL-C (mmol/L) | 1.09 (0.38) | 1.12 (0.37) | 1.04 (0.38) | 1.06 (0.38) | 1.06 (0.38) | 1.08 (0.38) | 1.11 (0.38) | 1.17 (0.38) | <0.001 |
| Apolipoprotein A ₁ (g/L) | 0.95 (0.15) | 0.96 (0.14) | 0.93 (0.15) | 0.93 (0.15) | 0.94 (0.14) | 0.95 (0.14) | 0.96 (0.14) | 0.98 (0.15) | <0.001 |
| Apolipoprotein B (g/L) | 0.87 (0.23) | 0.87 (0.23) | 0.87 (0.24) | 0.85 (0.23) | 0.87 (0.23) | 0.88 (0.23) | 0.88 (0.23) | 0.85 (0.23) | 0.38 |
| C-reactive protein (mg/L) | 3.7 (7.7) | 3.4 (7.2) | 4.4 (8.5) | 4.3 (7.8) | 3.7 (7.7) | 3.6 (7.7) | 3.5 (7.7) | 3.5 (7.7) | <0.001 |
| Albumin (g/L) | 39.7 (3.0) | 39.8 (2.8) | 39.3 (3.2) | 39.3 (2.9) | 39.6 (2.9) | 39.7 (2.9) | 39.9 (2.9) | 39.9 (2.9) | <0.001 |
| Fibrinogen (μmol/L) | 3.5 (0.8) | 3.5 (0.8) | 3.6 (0.9) | 3.6 (0.9) | 3.6 (0.8) | 3.5 (0.8) | 3.5 (0.8) | 3.5 (0.9) | 0.02 |
| Renal function | | | | | | | | | |
| eGFR (mL/min/1.73 m ²) | 72.2 (15.3) | 74.0 (14.4) | 68.6 (16.4) | 69.8 (14.7) | 71.7 (14.4) | 72.9 (14.4) | 73.0 (14.4) | 73.5 (14.5) | <0.001 |

Mean (SD), median (interquartile range), or *n* (%) shown.

25(OH)D, 25-hydroxyvitamin D; IHD, ischaemic heart disease (recall of diagnosis of myocardial infarction or angina); eGFR, estimated glomerular filtration rate; CVD, cardiovascular disease.

^aWith the exception of age and vitamin D, estimates are adjusted for age differences across vitamin D groups.

^{†b}Test of linear trend between log[25(OH)D] concentration and baseline characteristics after adjustment for age.

age-adjusted models (Figure 1). The shape of these associations were broadly similar for both vascular and non-vascular mortality [albeit with some attenuation of risk for non-vascular mortality with 25(OH)D concentrations above 80 nmol/L], and, for both outcomes, associations were consistent among men with and without a prior history of vascular disease, cancer, or diabetes.

Effect of adjustment for potential confounders

Given age, a doubling in 25(OH)D concentration [corresponding to a $\ln(2)$ absolute difference— ~ 2 SDs—on the log-scale] was, on average, associated with a 34% lower risk of vascular mortality (RR 0.66, 95% CI: 0.58–0.75) and a 36% lower risk of non-vascular mortality (RR 0.64, 95% CI: 0.58–0.72; Figure 2). After adjustment for prior diseases (including self-reported measures of health and frailty), established vascular risk factors, markers of inflammation and renal function, this was reduced to a 20% lower risk of vascular mortality (RR 0.80; 95% CI: 0.70–0.91) and a 23% lower risk of non-vascular mortality (RR 0.77; 95% CI: 0.69–0.86). The substantial change in the χ^2 statistics associated with 25(OH)D concentration with these adjustments (from 41.1 to 11.5 for vascular death and 63.3–21.4 for non-vascular death) suggest that a large part of

Table 2 Cause-specific mortality (annual death rate: % per year), overall and by prior disease

| | All men | No prior disease | Prior disease |
|----------------------------------|------------|------------------|---------------|
| Number of men | 5409 | 3568 | 1841 |
| Total follow-up (years) | 50 388 | 35 937 | 14 452 |
| Cause of death | | | |
| IHD | 659 (1.3) | 297 (0.8) | 362 (2.5) |
| Stroke | 378 (0.8) | 221 (0.6) | 157 (1.1) |
| Other vascular | 321 (0.6) | 209 (0.6) | 112 (0.8) |
| Subtotal: any vascular cause | 1358 (2.7) | 727 (2.0) | 631 (4.4) |
| Cancer | 809 (1.6) | 460 (1.3) | 349 (2.4) |
| Respiratory | 497 (1.0) | 321 (0.9) | 176 (1.2) |
| Other non-vascular | 551 (1.1) | 343 (1.0) | 208 (1.4) |
| Subtotal: any non-vascular cause | 1857 (3.7) | 1124 (3.1) | 733 (5.1) |
| Total: any cause | 3215 (6.4) | 1851 (5.2) | 1364 (9.4) |

IHD: ischaemic heart disease.

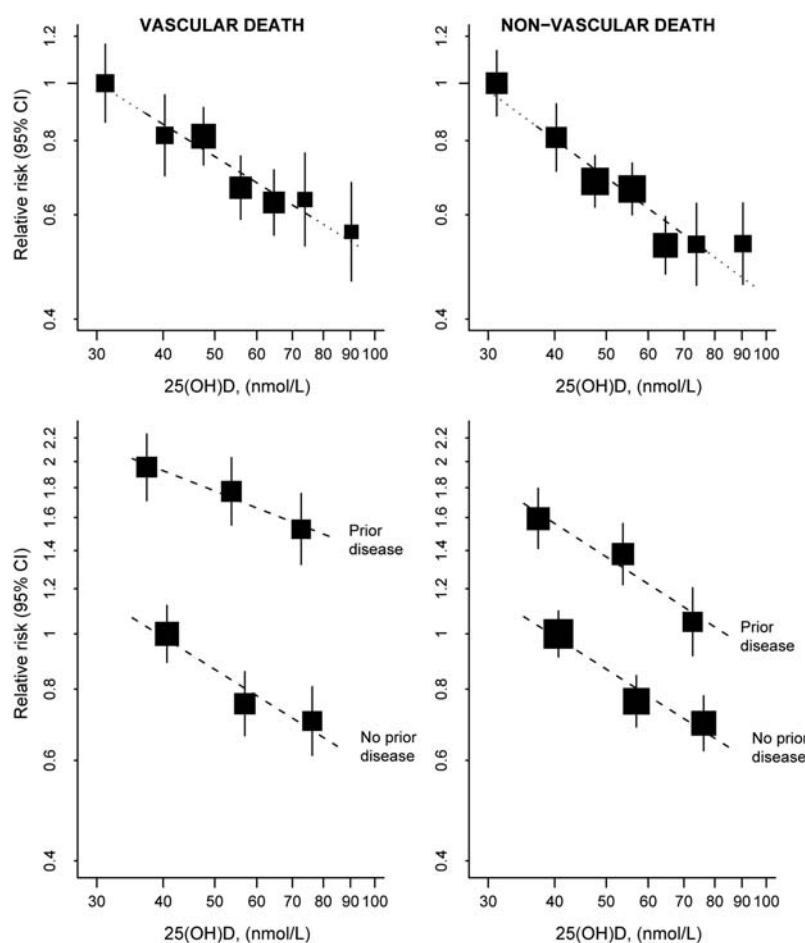


Figure 1 Age-adjusted relevance of measured 25-hydroxyvitamin D for vascular and non-vascular mortality in old age, overall, and separately in men with and without prior disease. Prior disease is defined as cardiovascular disease (recall of a diagnosis of myocardial infarction, angina, or stroke), diabetes, or cancer. In the lower panels, only three risk groups are shown for each disease category to increase the statistical reliability of such subgroup analyses. To convert 25-hydroxyvitamin D from nmol/L to ng/mL, divide by 2.496.

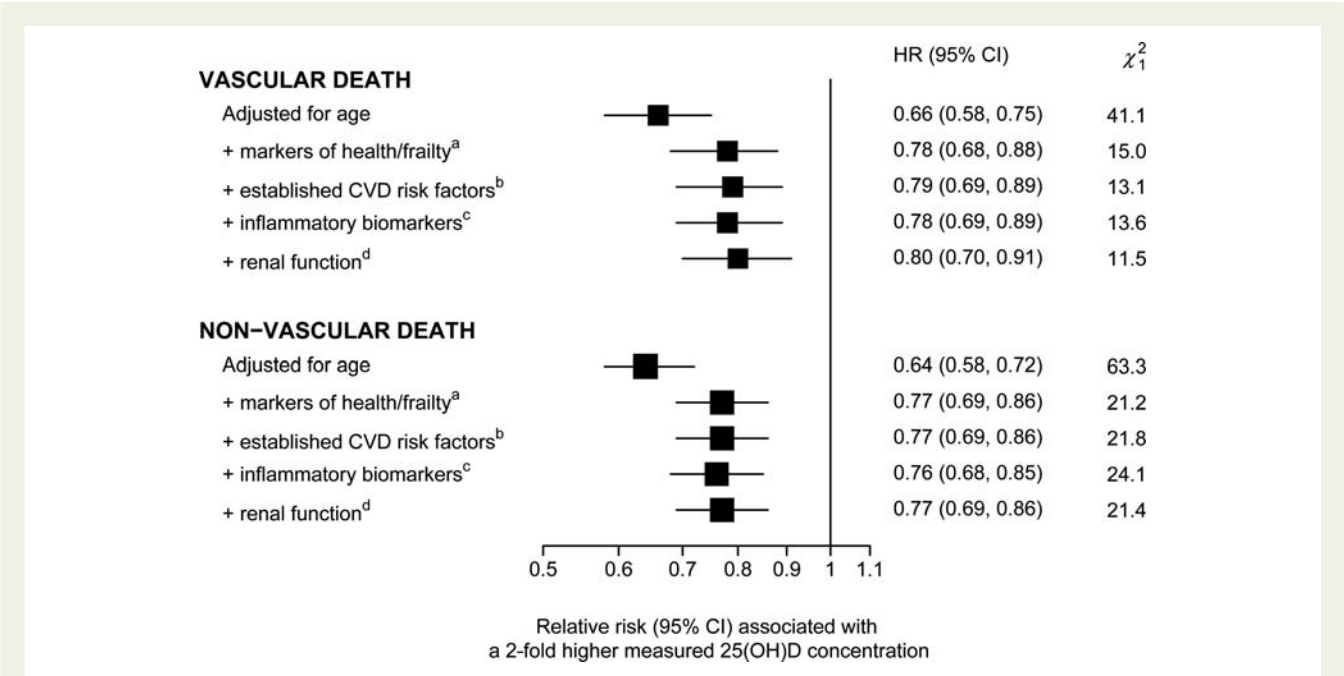


Figure 2 Effect of adjustment for known risk factors on the association between measured 25-hydroxyvitamin D and vascular and non-vascular mortality. (A) Recall of a diagnosis of ischaemic heart disease, stroke, cancer, or diabetes, plus self-reported health/frailty; (B) smoking status (current/ex/never); drinking status (current/non); grade of employment; LDL-C, HDL-C, apolipoprotein A₁, apolipoprotein B, body mass index, and blood pressure [recall (in 1997) of a diagnosis of hypertension, treatment (in 1997) for hypertension and measured systolic and diastolic blood pressure in both 1997 and in ~1970]; (C) albumin, fibrinogen, and C-reactive protein, and (D) estimated glomerular filtration rate.

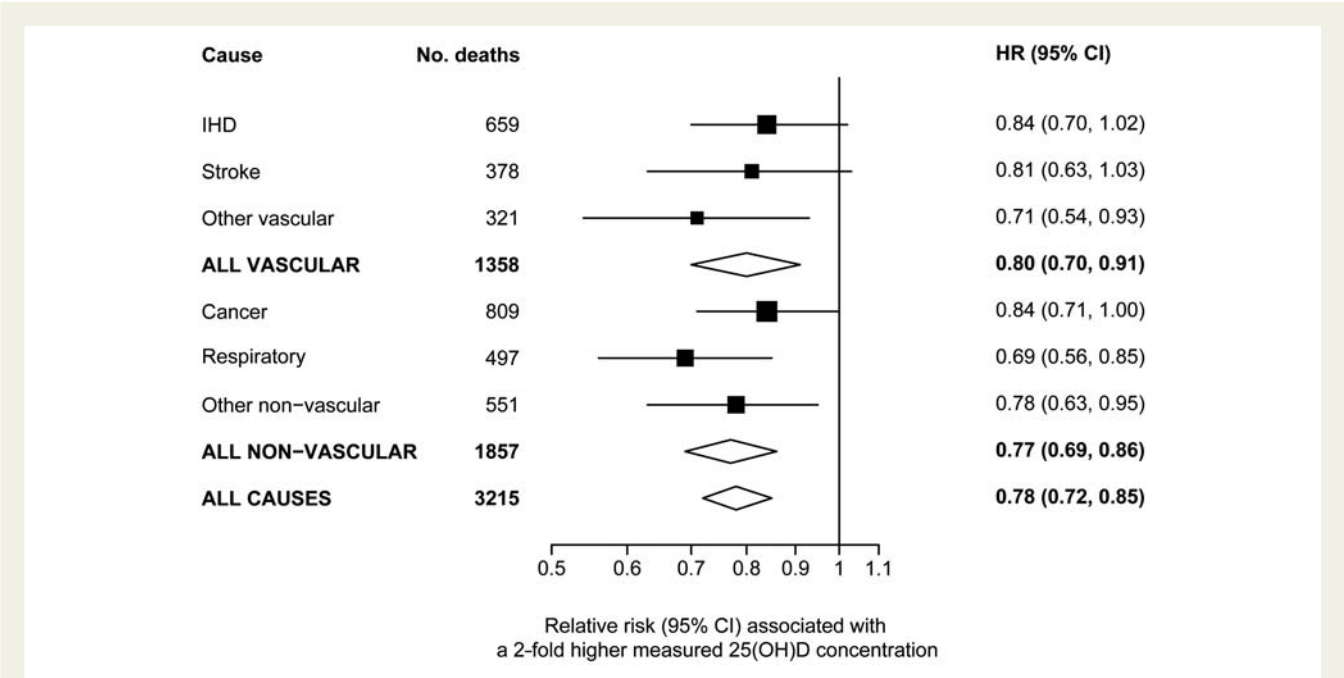


Figure 3 Association between measured 25-hydroxyvitamin D and cause-specific mortality after adjustment for measured confounders. IHD, ischaemic heart disease. Analyses are adjusted for smoking status (current/ex/never), drinking status (current/non), recall of a diagnosis of ischaemic heart disease, stroke, cancer, or diabetes, self-reported health/frailty, employment grade, LDL-C, HDL-C, apolipoprotein A₁, apolipoprotein B, body mass index, markers of inflammation (albumin, fibrinogen, and C-reactive protein), recall (in 1997) of a diagnosis of hypertension, treatment (in 1997) for hypertension and measured systolic and diastolic blood pressure in both 1997 and in ~1970, and estimated glomerular filtration rate.

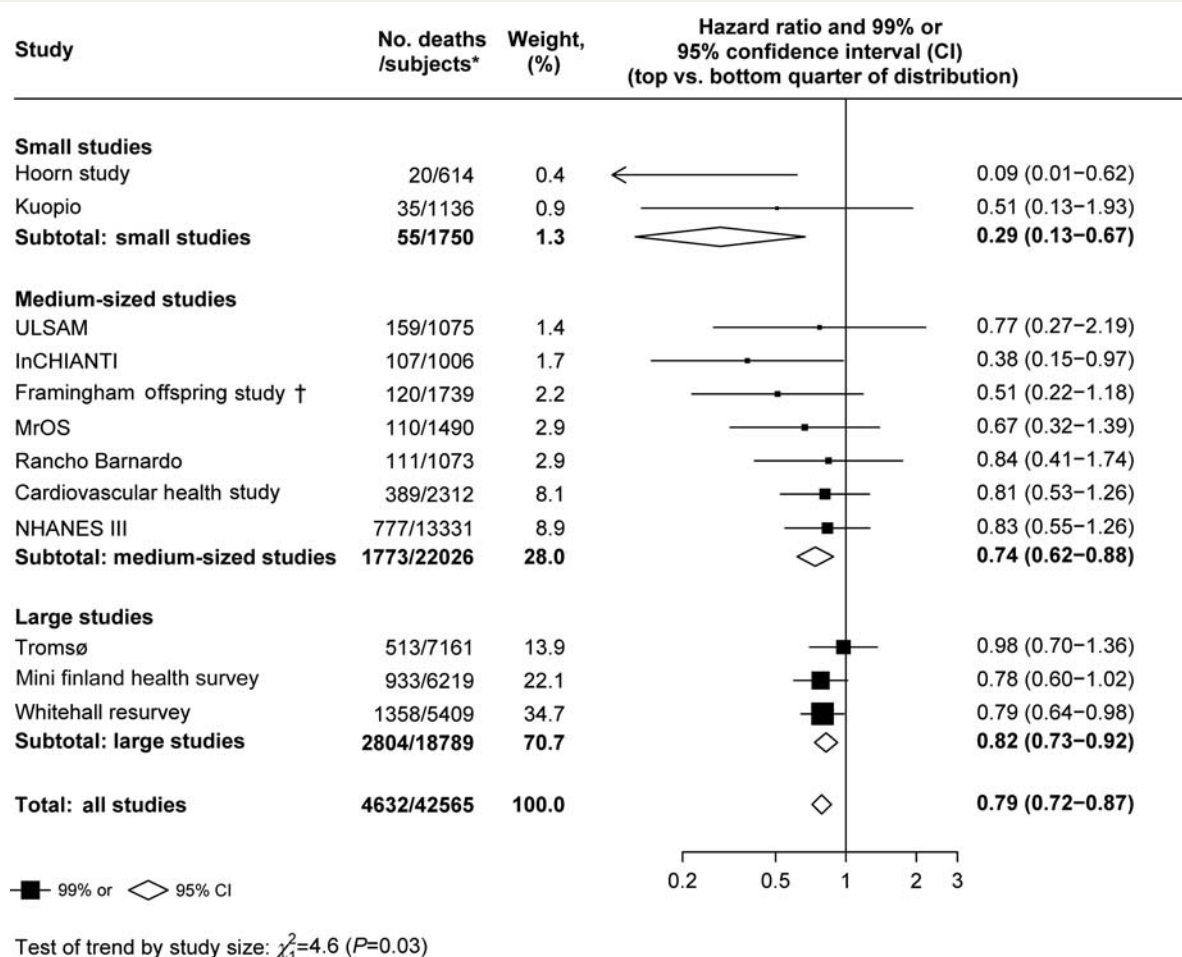


Figure 4 Meta-analysis of the relationship between 25-hydroxyvitamin D concentration and vascular mortality. *Numbers of deaths/people in the whole study are reported for each study, but only half of these deaths would be expected to contribute to analyses of top vs. bottom quarter. †This study included both fatal and non-fatal vascular events.

these associations was due to confounding, principally by prior disease. There was no evidence that the RRs associated with a doubling in baseline 25(OH)D concentration varied during follow-up (P values for test of proportionality assumption: $P = 0.48$ for vascular mortality and $P = 0.13$ for non-vascular mortality). Associations with particular types of vascular and non-vascular death (e.g. IHD, stroke, cancer, and respiratory death), after adjustment for measured confounders, were broadly similar to the overall RRs seen for vascular and non-vascular mortality (Figure 3). The findings for participants with no prior disease at resurvey were similar to those of the overall study population (see Supplementary material online, Figure S3 and S4). Results were also broadly similar after the exclusion of deaths within the first 5 years of follow-up (to further reduce the possible effect of reverse causality; see Supplementary material online, Figure S5) and were similar when the original 25(OH)D concentrations (i.e. before correction for seasonality) were used in analyses instead of season-adjusted concentrations (see Supplementary material online, Figure S6).

Meta-analysis of studies of 25-hydroxyvitamin D and vascular and all-cause mortality

The meta-analyses (which included results from the current study) included 12 prospective studies with 4632 vascular deaths and 18 prospective studies with 11 734 deaths from all causes. Participants with a 25(OH)D concentration in the top vs. bottom quarter of distribution had on average, 21% (95% CI: 13–28%) lower vascular mortality (Figure 4) and 28% (95% CI: 24–32%) lower total mortality (Figure 5). Observed RRs varied inversely with the amount of statistical information provided by each study (i.e. study size), with more extreme estimates being seen among smaller studies for both vascular and all-cause mortality.

Discussion

The Whitehall study, involving 3215 deaths, is one of the largest and longest prospective studies reporting associations of

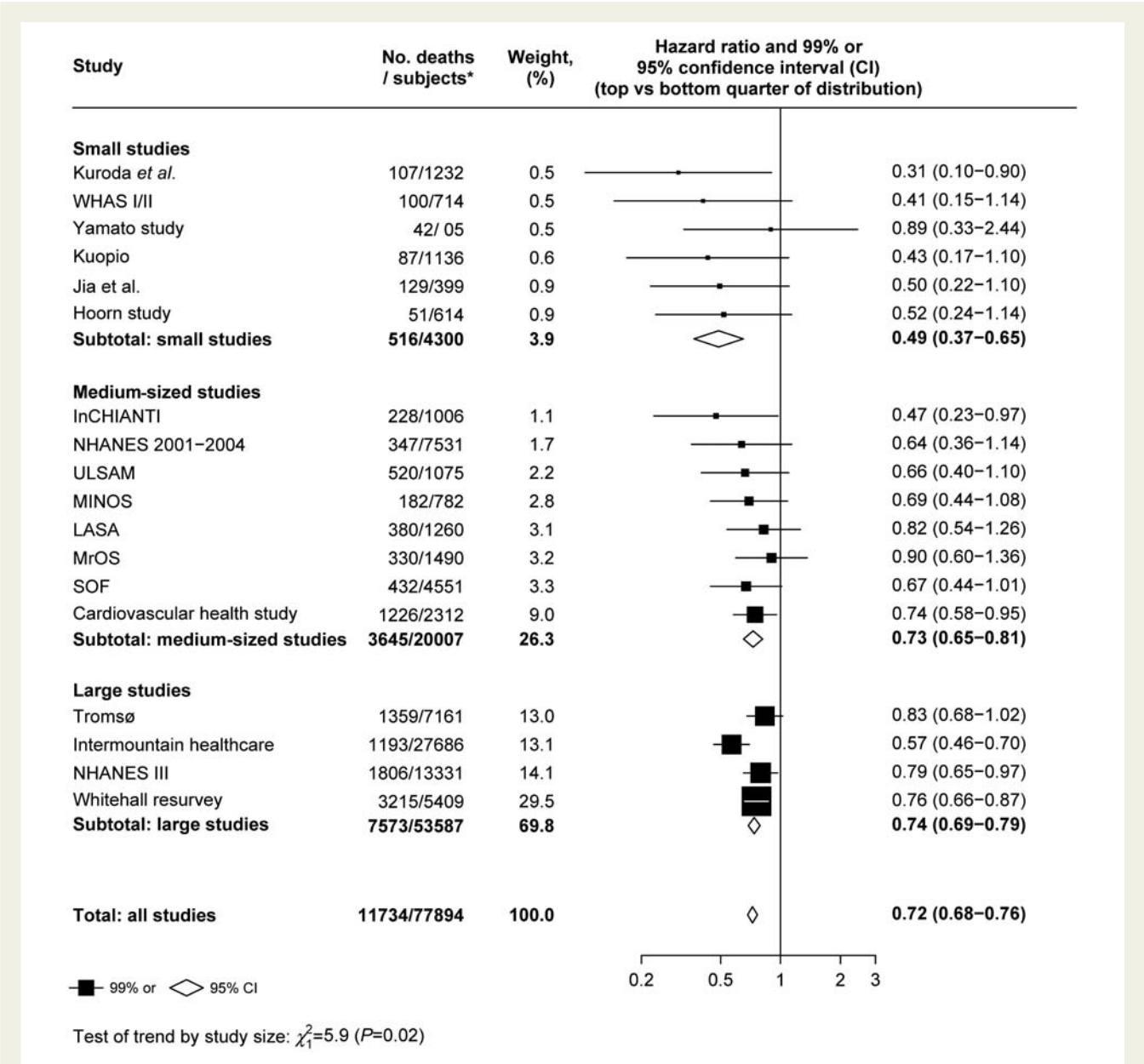


Figure 5 Meta-analysis of the relationship between 25-hydroxyvitamin D concentration and all-cause mortality. *Number of deaths/people in the whole study are reported for each study, but only half of these deaths would be expected to contribute to analyses of top vs. bottom quarter.

25(OH)D with cause-specific mortality, and the most informative study in the meta-analyses. This study showed an approximately linear (on the log–log scale) inverse association of plasma 25(OH)D concentration with both vascular and non-vascular mortality, at least within the range 30–90 nmol/L. After adjustment for age, seasonality, prior disease, markers of health and frailty, and other cardiovascular risk factors, a two-fold higher plasma concentration of 25(OH)D, achievable by supplementation with high doses of vitamin D, was associated with one-fifth lower risk of mortality (20% lower vascular mortality and 23% lower non-vascular mortality). The shape and strength of these associations

were broadly similar among men with and without prior vascular disease, cancer, or diabetes and persisted even after excluding deaths during the first 5 years of follow-up.

In contrast to previous meta-analyses that only reported on associations with all-cause mortality, the present analyses of prospective studies demonstrated a consistent trend in associations of 25(OH)D with vascular and all-cause mortality. Our analyses also showed a significant trend in effect size when the studies were ordered by size consistent with publication bias (where smaller studies are more likely to be published if their findings are strikingly positive than if they are negative or null). With

over 11 700 deaths, the current meta-analysis provides greater statistical precision than the recent meta-analysis involving 5562 deaths.²⁸

The similarities in the shape and strength of the associations of 25(OH)D with vascular and non-vascular causes of death observed in the present study may argue against a causal relationship with cardiovascular disease. It is possible that these associations may reflect incomplete adjustment for known risk factors that were measured imprecisely, or for unknown risk factors that may not have been measured. The possibility that associations could still reflect some reverse causality (despite the exclusion of deaths occurring within 5 years of blood collection in the present study) also cannot be entirely excluded.

Alternatively, the effects of vitamin D on vascular disease may be mediated by mechanisms that are independent of known cardiovascular risk factors. Increased vascular stiffness or vascular calcification may be one such mechanism, given the role vitamin D plays in calcium metabolism.²⁹ Moreover, as vitamin D receptors are found in a wide range of tissues, vitamin D may possibly influence diverse causes of death by some fundamental mechanism that is not yet fully understood. For example, vitamin D is believed to modulate the immune response which could influence deaths from cardiovascular and non-vascular causes, including cancer and infection.³⁰

Among the limitations of this study, data on prior disease and health status at resurvey were self-reported. Hence, while the strength of the associations were attenuated substantially after adjustment for these measures, it is possible that residual confounding by poor health status may persist. Formal assessments of physical activity were not made at baseline, but participants reported their self-rated health and ability to undertake activities of daily living (based on a 15-point questionnaire) and analyses were adjusted for these responses. Any non-response bias (by preferentially excluding frail older people) should have minimized rather than accentuated the effects of reverse causality.

Causes of deaths were supplied by the Office of National Statistics which may also have resulted in some misclassification of the causes of death in this population, which would tend to dilute any real differences between the different causes of death.³¹ Most men in this study were still living in and around the Greater London area. Although not representative of the wider population, this should reduce the likelihood of confounding by possible geographical factors associated with both hours of sunshine and mortality risk. Moreover, the results were broadly consistent with other studies included in the meta-analyses. Since individuals were classified on the basis of a single measurement of plasma 25(OH)D, it is possible that the true strength of the mortality associations observed with long-term average or usual 25(OH)D concentrations may be substantially steeper.³² The correlation between repeated measurements of log 25(OH)D recorded in 187 men over a 1.5 year period was 0.64. Hence, the mortality associations with long-term usual levels of 25(OH)D may be expected to be ~50% more extreme than those classified on the basis of single baseline measures.³²

Although our analyses of the observational studies have included strategies to minimize the effects of confounding and of reverse causality (within the limits of the study design), such studies are

unable to assess the causal relevance of 25(OH)D with vascular or non-vascular mortality. As yet, randomized trials have not been able to confirm or refute a causal role for vitamin D supplementation for either cardiovascular disease or cancer prevention. In a meta-analysis of 18 randomized-controlled trials, involving 57 311 participants, allocation to vitamin D for ~5.7 years was associated with a modest 7% lower overall mortality (RR 0.93, 95% CI: 0.87–0.99).¹⁶ In a recent Cochrane meta-analysis, involving nearly 11 000 deaths, allocation to vitamin D supplements did not significantly reduce mortality (RR 0.97, 95% CI: 0.94–1.00). Similarly, no beneficial effects of vitamin D supplements on risk of coronary heart disease or stroke were reported in the Women's Health Initiative (WHI) trial, in which 36 282 postmenopausal women were randomized to 400 IU vitamin D₃ daily vs. placebo.³³ Furthermore, in the RECORD trial of 5292 older people randomized to 800 IU vitamin D₃ daily vs. placebo, there was no evidence of any beneficial effects on mortality (RR 0.93; 95% CI: 0.85–1.02), or vascular disease (0.91; 95% CI: 0.79–1.05).³⁴

In the Whitehall study, the optimal concentration of 25(OH)D appeared to be ~80–90 nmol/L. While a recent meta-analysis reported an increased risk of mortality with concentrations above 87.5 nmol/L,²⁸ there were too few individuals with 25(OH)D levels greater than this in the present study to have sufficient statistical power to confirm or refute such an association. However, it is likely that larger doses of vitamin D, than those tested in previous trials, will be required to maintain concentrations of 25(OH)D > 80 nmol/L associated with the lowest risk in the observational studies.¹⁹ For example, among men in the lowest fifth of the 25(OH)D concentration in the present study, doses >2000 IU of vitamin D₃ daily may be required to double plasma 25(OH)D concentrations. Large trials are currently assessing whether daily supplementation with 2000–3000 IU of vitamin D₃ can reduce the risk of vascular disease, cancer, and other outcomes,³⁵ but it is unclear if even higher doses of vitamin D may be required to maintain blood concentrations of 25 (OH)D > 80 nmol/L throughout the year.

The reported inverse associations of 25(OH)D with higher risks of all-cause mortality, and now with vascular mortality, are of considerable public health interest, because low 25(OH)D concentrations are common in the population and may be easily reversed by supplements. However, the lack of specificity of the associations of 25(OH)D with particular causes of death in the present study casts doubt on the causal relevance of these associations. Large-scale trials using high doses of vitamin D supplements are required to determine whether such observed associations are causal and reversible or have other beneficial effects. Hence, it would be prudent to remain cautious about altering public health strategies to increase population mean levels of 25(OH)D pending the results of these trials.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

We would like to thank Jill Crowther for assistance in processing cause-specific mortality data, Sarah Clark and Jane Wintour for expertise on measurement of plasma concentrations of 25(OH)D, and Immunodiagnostic Systems for supplying free reagent. Dave Leon provided helpful comments on an earlier version of the paper.

Funding

The study was funded by the British Heart Foundation, BHF Cardiovascular Research Initiative, and the Medical Research Council, UK. The study was designed, undertaken, analysed, interpreted, and reported by the investigators independent of all funding sources.

Conflict of interest: none declared.

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Vitamin D and the risk of vascular and non-vascular mortality

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BACKGROUND

Moderate vitamin D deficiency (25 Hydroxy vitamin D, 25-OH D) affects about half the UK population and has been linked with increased risks of cardiovascular disease (CVD), cancer and impaired bone health and cognitive impairment (Figure 1). Limited vitamin D synthesis by the skin, due to inadequate exposure to sunlight, and inadequate dietary intake are the principal cause of these low levels.

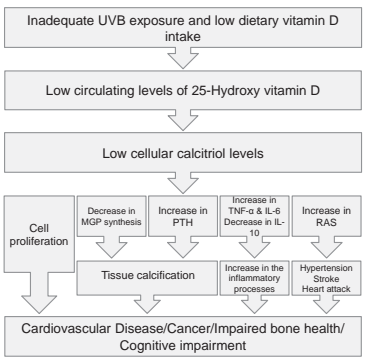


Figure 1: Possible pathways between Vitamin D deficiency and chronic disease, adopted ref. 1

Whether vitamin D deficiency is a consequence of disease or a contributing factor is not clear as chronic illnesses may affect access to sunlight and diet. Large scale population studies may address this question, however, the gold standard method for the measurement of 25-OH D, LC-MS/MS is expensive, time-consuming and not suitable for high-throughput testing.

Alternative automated immunoassay methods are available and we have investigated the IDS-iSYS system for the analysis of 25-OH D.

THE WHITEHALL RE-SURVEY STUDY

The 1967-1970 Whitehall Study investigated social determinants of health among 18000 British male civil servants, with specific information on cardiovascular disease and mortality rates². The Whitehall re-survey is a follow-up study (1997) with stored plasma from 5500 surviving participants (mean age 75 years).

Moderate vitamin D deficiency has been linked with higher risk of myocardial infarction, stroke and death from cardiovascular disease, however, whether this association is causal is unknown.

Using the Whitehall resurvey samples, we have examined the relevance of circulating 25-OH D levels to vascular mortality and also non-vascular mortality.

AIM

- To describe a formal validation of the IDS-iSYS 25-OH D assay.
- To assess the relationship of 25-OH D with vascular and non-vascular mortality in the Whitehall prospective study of older men.

METHODS

Assay validation

Assay validation is the process of demonstrating that analytical procedures are suitable for their intended use.

The assay performance is judged by comparison to recommendations for the types and size of analytical errors that are allowable; without invalidating the usefulness of the test results.

The following elements were assessed for the IDS-iSYS 25-OH D assay:

i. Accuracy (Figure 2a)

An assessment of how close the IDS-iSYS system is to obtaining a true 25-OH D value was made using NIST reference material and proficiency testing.

ii. Precision (Figure 2a)

The reproducibility of repeated 25-OH D measurements were investigated at three levels of sample concentration for 25-OH D for both within and between batch precision.

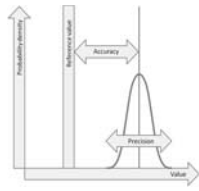


Figure 2a: Accuracy and Precision.

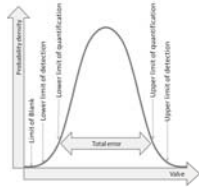


Figure 2b: Functional Sensitivity.

iii. Functional Sensitivity (Figure 2b)

The lower limit of the working range was estimated as the mean concentration for a sample that has a between batch CV = 20%.

iv. Recovery

The proportional systematic error was determined from pairs of samples, the first sample analysed as a reference, the second sample spiked with a high concentration of 25-OH D.

v. Linearity

The linearity across the normal population reference range (10-50ng/ml)³ for 25-OH D was investigated.

Measurement of 25-OH D in Whitehall samples

Whitehall samples (n=5500) were analysed for 25-OH D in batches of 40 using the automated IDS-iSYS system.

Internal quality checks included running control samples with each batch, paired samples, which were blinded to operator, across the population group and re-testing 5% of samples.

RESULTS

Accuracy

Investigation of NIST standard reference material 968

The NIST reference material was analysed in triplicate. The mean, coefficient of variation and recovery was calculated and compared to the manufacturer's allowable limits⁴ (Table 1).

Table 1. Comparison of observed results with NIST

| Sample | Mean (ng/ml) | Expected CV | Observed CV | Recovery |
|---------|--------------|-------------|-------------|----------|
| Level 1 | 22.74 | 5.5 | 3.43 | 95% |
| Level 2 | 19.72 | 5.5 | 13.19 | 140% |
| Level 3 | 35.74 | 5.5 | 3.32 | 79% |
| Level 4 | 36.13 | 5.5 | 5.7 | 102% |

A significant over recovery for level 2 is observed which is explained by the use of horse serum in the preparation of this sample.

Investigation of DEQAS EQA samples

DEQAS EQA samples were analysed in duplicate and the results compared to DEQAS data (Figure 3). The observed results show a mean positive bias of 29.9% against the all methods mean which is within the acceptable limits specified by DEQAS. Compared to the LC-MS/MS a positive bias of 21.8% was observed.

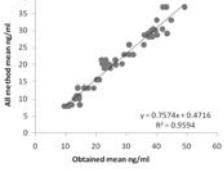


Figure 3: Correlation of observed results against DEQAS data

Precision

Within batch precision: Different samples were selected that represented low, medium and high concentrations for Vitamin D and analysed within the same run (Table 2).

Between batch precision: Different samples were selected that represented low, medium and high concentrations for Vitamin D and analysed across multiple runs (Table 3).

The mean and coefficient of variation was calculated and compared to the manufacturers claims⁵.

Table 2: Within batch precision results.

| Sample | Mean (ng/ml) | Expected CV | Observed CV | N |
|--------|--------------|-------------|-------------|----|
| Low | 6.21 | 12.1 | 12.91 | 20 |
| Mid | 27.15 | 5.5 | 4.63 | 20 |
| High | 58.81 | 7.3 | 2.82 | 20 |

Table 3: Between batch precision results.

| Sample | Mean (ng/ml) | Expected CV | Observed CV | N |
|--------|--------------|-------------|-------------|----|
| Low | 13.27 | 16.9 | 12.68 | 47 |
| Mid | 32.79 | 10.4 | 8.34 | 47 |
| High | 63.19 | 8.9 | 7.55 | 48 |

Functional Sensitivity

The mean concentration for a sample whose between batch CV crosses the 20% threshold is approximately 5.2ng/ml (Figure 4). The manufacturer claims the lower analytical range of the assay to be 5ng/ml.

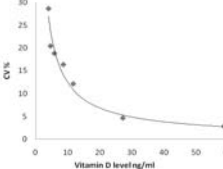


Figure 4: Relationship of sample concentration against between batch CV

Recovery

The mean % recovery was 98.47% with a SD of 7.49. This equates to a proportional systematic error of 1.53%. The DEQAS evaluation criteria for routine assessment allows a bias of 10%⁶. Recovery is therefore acceptable.

Linearity

Six plasma pools derived from mixing proportional amounts of low vitamin D level plasma and high vitamin D level plasma sample material were analysed in quadruplicate. The recoveries were calculated to be between 104.36% and 111.14%. These are comparable to the manufacturers claims (102 – 107%).

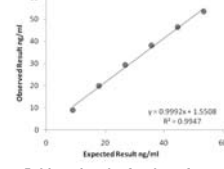


Figure 5: Linearity plot for the reference range.

Whitehall sample results

Measurement of the Whitehall study samples showed a log linear inverse relationship between plasma 25-OH D levels with vascular and non-vascular mortality throughout range. A 2-fold higher plasma level of 25-OH D was associated with approximately 25% lower relative risk of vascular and non-vascular death (Figure 6).

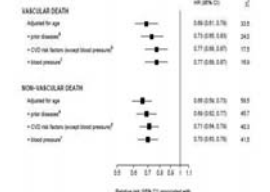


Figure 6: Relative risk plot

CONCLUSIONS

- The IDS iSYS 25-OH D assay was shown to be suitable for measurement of samples from the Whitehall study.
- The Whitehall results show an inverse association between vitamin D levels and the risk of vascular and non-vascular mortality. This has led to a further study to investigate the safety and efficacy of high dose vitamin D and clarify the relevance of vitamin D in chronic disease in older people.

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²Journal of Epidemiology and Community Health, 1978, 32, 244-249 Employment grade and coronary heart disease in British civil servants
M. G. Marmot, Geoffrey Rose, M. Shipley, and P. J. S. Hamilton

³ Tietz textbook of clinical chemistry, second edition.
⁴ NIST Certificate of Analysis SRM 972 Vitamin D in Human Serum.
⁵ IDS-iSYS 25-Hydroxy Vitamin D assay Kit and Controls insert sheets
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D. Stockl et al / Clinica Chimica Acta 408 (2009) 8-13

Research funded by:



Estimation of the optimum dose of vitamin D for disease prevention in older people: rationale, design and baseline characteristics of the BEST-D trial

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(ISRCTN Number 07034656; EudraCT Number 2011-005763-24)

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Conflict of interests: Robert Clarke, Connie Newman, Joseph Tomson, Harold Hin, Rijo Kurien, Jolyon Cox, Michael Lay, Jenny Sayer, Michael Hill, Jonathan Emberson, Jane Armitage declare that they have no conflict of interest.

Running title: Dose-finding trial of vitamin D

Word count: Abstract: 294; Text: 2797 plus 2 tables and 1 figure and 31 references

Funding: We are grateful to Tischcon Corporation (Westbury, New York, USA) who kindly donated the active and placebo vitamin D capsules. The British Heart Foundation (PG/12/32/29544) and British Heart Foundation Centre for Research Excellence provided partial funding for the study. The Clinical Trial Service Unit and Epidemiological Studies Unit (CTSU) at the University of Oxford received funding from the UK Medical Research Council, the British Heart Foundation and Cancer Research UK.

Abstract

Background: Previous large trials of vitamin D for prevention of fractures and other disease outcomes have reported conflicting results, possibly because the doses tested were insufficient to maintain optimum blood levels of vitamin D (25[OH]D) predicted by the observational studies.

Methods: The present report describes the design and baseline characteristics of a trial comparing the biochemical and other effects of daily dietary supplementation with vitamin D in preparation for a large trial for the prevention of fractures, cardiovascular disease and cancer.

Methods: The trial will compare the biochemical and other effects of daily dietary supplementation with 100µg or 50µg vitamin D3 or placebo, when administered for 12 months, in 305 ambulant community-dwelling older people living in Oxfordshire, England. The primary analyses will compare 12-month mean plasma concentrations of 25(OH)D as well as the proportion of participants with a 12-month concentration >90 nmol/L between participants allocated 100µg and participants allocated 50µg daily. Secondary analyses will compare the two active doses (both separately and when combined) with placebo. Additional end-points include biochemical assessments of safety, blood pressure, arterial stiffness, falls, fractures, heel and wrist bone density, grip strength and physical performance and echocardiographic assessments of cardiac function in a random sample of participants.

Results: About one-third of eligible participants agreed to participate in the trial. The mean age was 72 (SD 6) years with equal numbers of men and women. About one third reported a prior history of fracture or hypertension, one-fifth reported a prior

cardiovascular event, and one tenth reported diabetes or a fall in the previous 6 months.

Conclusions: The results of this trial will help determine the optimum dose of vitamin D to test in a larger trial investigating whether vitamin D supplementation can reduce the risk of fractures, cardiovascular disease or cancer.

Keywords: Vitamin D supplementation trial, 25-hydroxyvitamin D, parathyroid hormone.

Introduction

Osteoporosis causes substantial morbidity and mortality in older people, but whether chronic insufficiency of vitamin D is a reversible determinant of osteoporosis and related risk of fractures is controversial. Observational studies indicate that low plasma levels of 25-hydroxyvitamin D [25(OH)D] are associated with higher risks of fractures and with vascular and non-vascular mortality (1-4), but it is unclear if these associations are causal. Randomized trials assessing the effects on fracture and other health outcomes have generally failed to demonstrate beneficial effects of vitamin D supplementation (5-14). However, few such trials have used sufficient doses of vitamin D3 to achieve and maintain what might be considered optimum plasma levels of 25(OH)D.

Although controversial, the available evidence suggests that the optimum plasma levels of 25(OH)D may be around 75 to 90 nmol/L. Firstly, parathyroid hormone (PTH) levels are linearly and inversely associated with plasma 25(OH)D levels until such levels reach about 75 nmol/L (15, 16). Secondly, prospective observational studies indicate that the risks of vascular and non-vascular mortality are lowest at plasma 25(OH)D levels of around 90 nmol/L (4). Thirdly, mean peak plasma 25(OH)D levels at the end of summer in young British adults are also about 90 nmol/L (17). At plasma 25(OH)D levels below a cut-off point of about 75 nmol/L, the average increase in 25(OH)D per 10µg of additional vitamin D3 has been estimated to be about 7-10 nmol/L (16, 18). Hence, from a typical level of 25(OH)D of 55 (SD 26) nmol/L found in older UK adults (19), a dose of vitamin D3 of at least 50µg (2000 IU), and possibly as much as 100µg (4000 IU), may be required to achieve and maintain blood levels >90 nmol/L throughout the year in most people in the UK (20).

The aims of the BEST-D trial are to determine the optimum dose of vitamin D that is safe and effective to test in a large trial of vitamin D for prevention of fractures, vascular disease and cancer in older people living in the community. The primary objectives are to compare the effects on blood concentrations of 25(OH)D and the proportion of participants with 25(OH)D concentrations >90 nmol/L after one year of daily supplementation with 100 µg (4000 IU) or 50 µg (2000 IU) vitamin D3 versus placebo. The aim of the present report is to describe the rationale and design of the BEST-D trial and the baseline characteristics of the trial participants.

Methods

Inclusion and exclusion criteria

Ambulant community-dwelling men and women aged 65 years and older were identified from a single general practice in Oxfordshire and mailed an invitation letter to participate in the trial. The invitation letter included a screening questionnaire to check eligibility and a study information leaflet. Those who returned the screening questionnaire and were potentially eligible were provided with an appointment for a randomization study visit. **Figure 1** outlines the number of participants enrolled in each stage in the study.

People were ineligible if they were: nursing home residents; regular users of vitamin D supplements more than 10 µg (400 IU) vitamin D daily; prescribed calcium supplements, bisphosphonates, parathyroid hormone (PTH), or calcitonin; had medically diagnosed dementia or history of hypercalcaemia, hyperparathyroidism

lymphoma, sarcoidosis, active tuberculosis or renal calculus; were judged by their doctor as likely to be poorly compliant with clinic visits or medication; or had a history of alcohol or substance misuse or a history that might limit their ability to take the study treatment (e.g. terminal illness). All participants provided written informed consent.

Outcomes

The co-primary outcomes are mean plasma 25(OH)D concentrations at 12 months and the percentage of participants with a 12-month 25(OH)D concentration >90 nmol/L. Secondary outcomes are: (i) mean plasma 25(OH)D concentrations at 1 and 6 months; (ii) percentage of participants with plasma 25(OH)D concentrations >90 nmol/L at 1 and 6 months; (iii) PTH within the reference interval (1.1-6.8 pmol/L) at 1, 6 and 12 months; (iv) albumin-corrected calcium concentrations above the reference interval (2.15-2.55 mmol/L) at 1, 6 and 12 months; and (v) mean plasma concentrations of albumin, phosphate, creatinine and alkaline phosphatase at 6 and 12 months; (vi) mean concentrations of lipids (total cholesterol, LDL-C, HDL-C, triglycerides, apo-B and apo-A1), high-sensitivity C-reactive protein (hsCRP), and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) at 6 and 12 months; (vii) mean levels of systolic and diastolic blood pressure, heart rate and arterial stiffness at 6 and 12 months; and (viii) echocardiographic measures of systolic function, diastolic function and global cardiac function in a sub-set of 150 participants at 12 months. Tertiary outcomes are listed in the Web-Appendix, and include 12-month assessments of: (i) reported fractures at all sites and at specific sites; (ii) reported falls; (iii) muscle and joint pain; (iv) grip strength; (v) Short Physical

Performance battery; (vii) Geriatric Depression Scores; (viii) respiratory infections; (ix) Height, weight and BMI; (x) bone density of wrist and heel; and (xi) estimated GFR and urinary albumin/creatinine ratio.

Sample size and statistical analyses

Based on the reported mean seasonally adjusted 25(OH)D levels of 55 (SD 20) nmol/L among post-menopausal women between the ages of 65 and 74 years (21), a dose of 50 µg of vitamin D3 may be required to increase the mean concentration of 25(OH)D by 25-35 nmol/L. However, there is uncertainty about whether the relationship between vitamin D dose and the increase in 25(OH)D is linear. The study was designed to have good power to detect even a small difference in mean 25(OH) concentrations at 12 months between the two active doses. Specifically, with 100 participants in each group and an assumed SD of 20 nmol/L, this study has 90% power (at $2p=0.01$) to detect a true difference in mean 25(OH)D between the two active doses at 12 months of just 11 nmol/L. (The minimum detectable difference will be even smaller than this due to the use of analysis of covariance to assess comparisons: see Web-Appendix.)

The primary efficacy assessment will be an “intention-to-treat” analysis among all randomized participants comparing daily dietary supplementation with vitamin D3 100µg vs vitamin D3 50µg on: (1) the mean 25(OH)D concentrations at 12 months and (2) the proportion of individuals with levels of 25(OH)D above 90 nmol/L at 12 months. Pre-specified subgroup analyses of the co-primary outcomes will include subdivisions by: sex; age (<70 vs ≥70 years); higher or lower body mass index (BMI); baseline plasma concentrations of 25(OH)D; estimated glomerular filtration

rate; and estimated dietary calcium intake; or presence or absence of prior cardiovascular disease or cancer. No allowance will be made for multiple comparisons in the assessment of the co-primary endpoints with the statistical significance of each being based on a $2p < 0.05$ criterion.

Secondary and tertiary analyses of particular outcomes will compare the two active doses (separately and/or when combined) with placebo. (A detailed Statistical Analysis Plan is provided in the Web-Appendix). Allowance will be made in their interpretation for multiple hypothesis testing (taking into account, if relevant, the type of measure and evidence from other studies) (22, 23). But, the more extreme the p-value (or, analogously, the further the confidence interval is from zero) after any allowance has been made for the nature of the particular comparison, the more reliable the comparison and, hence, the more definite any finding will be considered.

Randomization and blinding

Table 1 shows a summary of the study procedures. Study visits were conducted by trained study nurses at the participant's home (but the final visit also included an additional visit to the General Practice: see below). At the first visit (month 0), eligibility was assessed and relevant medical history and medication, calcium intake, and baseline measures recorded. If willing and eligible, participants were randomized in a ratio of 1:1:1 to vitamin D3 100 µg (4000 IU), vitamin D3 50 µg (2000 IU), or placebo daily. Randomization was performed by a central randomization service at the Clinical Trial Service Unit (CTSU), University of Oxford using a minimisation algorithm balanced for age group [65-69, 70-74, 75+ years], gender, body mass index (BMI), smoking history, ethnicity and history of fracture to

assign a double-blind treatment allocation and a treatment allocation number. Vitamin D3 50 µg in soft gel capsules and matching placebo capsules were provided by Tischcon Corporation (Westbury, New York, USA), and packaged in labelled child-proof bottles by Sharp Clinical Services (Crickhowell, Powys, UK). At the randomization and 6 month visits, participants were provided with two bottles of medication, each containing 210 capsules (each containing 50 µg vitamin D3 or matching placebo) sufficient to last 7 months, and instructed to take one capsule from each bottle daily. Participants were asked to return all study treatment bottles at the 6 and 12 month visits.

Post-randomization follow-up

About 100 participants were randomly selected for a blood sample at 1 month (Table 1). All participants were to have follow-up visits from the study nurse at 6 and 12 months (Table 1). Compliance and adverse events were evaluated at each visit along with selected tests. Depression scores were assessed at 6 and 12 months using a 4-point Geriatric Depression Score (24). Spot urine samples were collected at the final home visit. Participants were invited to attend a special clinic at their local General Practice for a bone scan to assess heel and wrist bone density and a short physical performance battery (25).

Safety

At each visit non-serious adverse events (NSAEs) thought related to the study treatment or that resulted in the participant stopping treatment, and all serious

adverse events (SAEs) were recorded in the electronic case report forms. Participants were provided with a 24-hour Freefone number to contact a clinician at CTSU should they wish to discuss trial-related medical problems.

Cardiovascular assessments

At each visit blood pressure and arterial stiffness were measured after 10 minutes of rest in the seated position (26). A finger probe (Pulsetrace PCA 2) placed on the right forefinger recorded the 'digital volume pulse' using photoplethysmography over 30-60 seconds. This was followed by blood pressure and brachial artery arterial stiffness measurements made over 2 minutes using a TensioClinic™® arteriograph. Left ventricular function was assessed by echocardiography in a randomly selected subset of 151 participants at the final visit (who provided separate consent for this examination). Any significant abnormalities were to be reported to the participant's GP.

Musculoskeletal parameters

A history of falls in the last 6 months and fractures at any time was recorded at randomization, 6 and 12 months. Participants were asked to rate from 1 to 10 their level of physical activity, and the presence and severity of any muscle and, separately, joint pain, if present. Hand grip strength was assessed using a Jamar™ J00105 hydraulic dynamometer, as previously described (27). A modified short physical performance battery at the final visit evaluated the time taken for five consecutive chair rises, the time to walk 3 metres, and a balance score calculated from the time that balance was held in the tandem, semi-tandem, and side-by-side

stances (25). Heel and wrist bone mineral density were measured using an OsteoSys EXA-3000 scanner (OsteoSYS, Seoul, Korea).

Laboratory analyses

Blood samples were collected at each visit into 2 standard 10 ml tubes (one containing EDTA and one lithium heparin) and at baseline and 12 months also into a PAXgene blood RNA tube (Pre-Analytix, Qiagen, Hombrechtiken, Germany). Samples were taken to the Horton Hospital Clinical Chemistry laboratory, Banbury, Oxfordshire, processed, fractionated into aliquots within 4 hours of blood collection and stored in a -80°C freezer. Lithium heparin plasma was used to measure plasma levels of calcium and albumin at the Horton Hospital Clinical Chemistry laboratory prior to starting study treatment. Plasma samples were stored at the Wolfson Laboratory in CTSU, Oxford for future determination of plasma levels of PTH, 25(OH)D, lipids, hsCRP and inflammatory cytokines at CTSU. Buffy coats were also stored from the randomization and 12 month visits for future extraction of DNA to measure markers related to vitamin D. Urine samples were collected at the final visit and frozen (-80 °C) for future measurement of urinary albumin to creatinine ratio.

Data collection and management

All questionnaire and clinical data were collected electronically using portable computers and encrypted to ensure confidentiality, data accuracy and protection of patient safety. BEST-D was conducted in accordance with the principles of the

International Conference on Harmonisation Guidelines for Good Clinical Practice (ICH-GCP), and relevant regulations.

Ethics committee approval and funding

BEST-D received approval from the National Research Ethics Service (NRES) Committee South Central – Oxford B, the Thames Valley Primary Care Research Partnership, a Clinical Trial Authorisation from MHRA and is included on the National Institute for Health Research (NIHR) Trial portfolio. The University of Oxford acted as sponsor and funding was provided by the Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford and the British Heart Foundation.

Results

Characteristics of study participants

From the registered patients at the practice in this age group, 932 were invited to participate, 313 (33%) agreed to have a randomization visit from the study nurse and 305 participants were successfully randomized between 24 September 2012 and 14 March 2013 (**Figure 1**). Eight participants visited were not randomized (6 declined and 2 were ineligible) and 619 declined or ignored the invitation to participate. The final study visit for the last participant was held on 10 March 2014. Among surviving participants, 296 completed a 6-month visit, 290 completed a 12-month visit, 272 had their bone density measured and 151 had cardiac echocardiography.

Baseline characteristics

Among the 305 participants, the overall mean age was 72 (SD 6) years, 51% were men, 14% had a prior history of IHD, 6% had stroke/TIA, 9% had diabetes and 39% had hypertension. Overall, 30% had any prior history of fracture and 13% had a history of a fall in the last 6 months. Table 2 also shows the use of medication for prevention of cardiovascular disease, mean levels of body mass index, blood pressure and grip strength; and estimated calcium intake. While use of anti-hypertensive drugs, anti-platelet agents and statins were appropriate for the proportions with prior vascular disease and diabetes in this general population, routine use of non-prescribed (“over-the-counter”) preparations of calcium and vitamin D supplements was uncommon (albeit this partly reflected the study design).

Discussion

Although severe vitamin D deficiency is associated with rickets in children and osteomalacia in adults (28), there is no consensus about routine supplementation with vitamin D for bone health. The UK dietary Reference Nutrient Intake (RNI) for vitamin D is 10 µg/day (400 IU) for adults aged 65 years and older and pregnant or lactating women. The RNI has not been established for otherwise healthy adults < 65 years old because of the assumption that sufficient vitamin D can be made after exposure to sunlight.

In 1992, Chapuy et al (5) reported that supplementation with 20 µg (800 IU) vitamin D plus calcium 1200 mg daily for 18 months significantly reduced non-vertebral fractures (160 vs 215; $p < 0.001$) including hip fractures (80 vs 110; $p = 0.004$) in

3,270 elderly women living in nursing homes or apartments for elderly people in France. Over the last 20 years, several large, long-term trials, and several study-level and individual participant-level meta-analyses have assessed the effect of vitamin D on fractures, but the results have been conflicting (6-14), possibly because the doses of vitamin D used were insufficient to maintain optimum levels of 25(OH)D. However, there have been few large trials of vitamin D that used adequate doses of vitamin D to achieve the blood levels of 25(OH)D that were predicted by the observational studies to be associated with lowest risk. While there is no consensus about the optimum levels of 25(OH)D for bone health, the available evidence suggests that it may be close to 90 nmol/L, the level reached by healthy UK adults at the end of the summer (17), and below which PTH levels may be elevated (15, 16) and the level in observational studies associated with the lowest risk of vascular and non-vascular mortality (4).

BEST-D is the first randomized placebo-controlled trial to assess the effects of two higher daily doses of vitamin D, 100 µg and 50 µg (equivalent to 4000 IU and 2000 IU), in older adults living in the UK, on plasma levels of 25(OH)D, PTH, calcium, phosphate and albumin. An additional advantage of choosing 4000 IU daily is to maximize mean levels and the proportion greater than 90 nmol/L of 25(OH)D levels after taking account of anticipated non-compliance of about 10-15% with allocated study treatment. The detailed clinical and biochemical monitoring at 0, 6 and 12 months should provide reliable data on the safety of higher doses of vitamin D. The results of this trial should establish the optimum daily dose of vitamin D to be used in future randomized trials in the UK that evaluate whether vitamin D supplementation confers the musculoskeletal and other health benefits suggested by observational studies (29-31).

Appendix: BEST-D Collaborative Group

Writing Committee: Robert Clarke^{1,*}, Connie Newman^{2,*}, Joseph Tomson^{1,*}, Harold Hin^{3,*}, Rijo Kurien¹, Jolyon Cox¹, Michael Lay¹, Jenny Sayer¹, Michael Hill¹, Jonathan Emberson¹, Jane Armitage^{1,*} (*contributed equally).

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². Division of Endocrinology & Metabolism, New York University School of Medicine, New York, USA.

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Steering Committee: Jane Armitage (Chief Investigator and Chair), Harold Hin and Robert Clarke (Lead Investigators), Michael Hill (Laboratory Director), Joseph Tomson, Connie Newman, and Richard Haynes (Clinician Members), Anne Dawson (Lay Member), Jonathan Emberson (Statistician), Michael Lay (Computing) and Jenny Sayer (Administrative Coordinator).

Medication: Kevin Murphy.

Computing: Michael Lay, Jolyon Cox, Rijo Kurien.

Clinical Biochemistry, Horton Hospital, Banbury: Tim James and Peter Tandy and Wolfson Laboratory, Clinical Trial Service Unit, Oxford: Michael Hill

Nurses: Lynn Peach, Enid Frost, Caroline Boulton, Barbara White.

Research Assistant: Jessica Hin.

Echocardiography: Joseph Tomson, Linda Arnold.

Administrators: Kate Gillingham, Jenny Sayer.

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Table 1: Schedule of study procedures

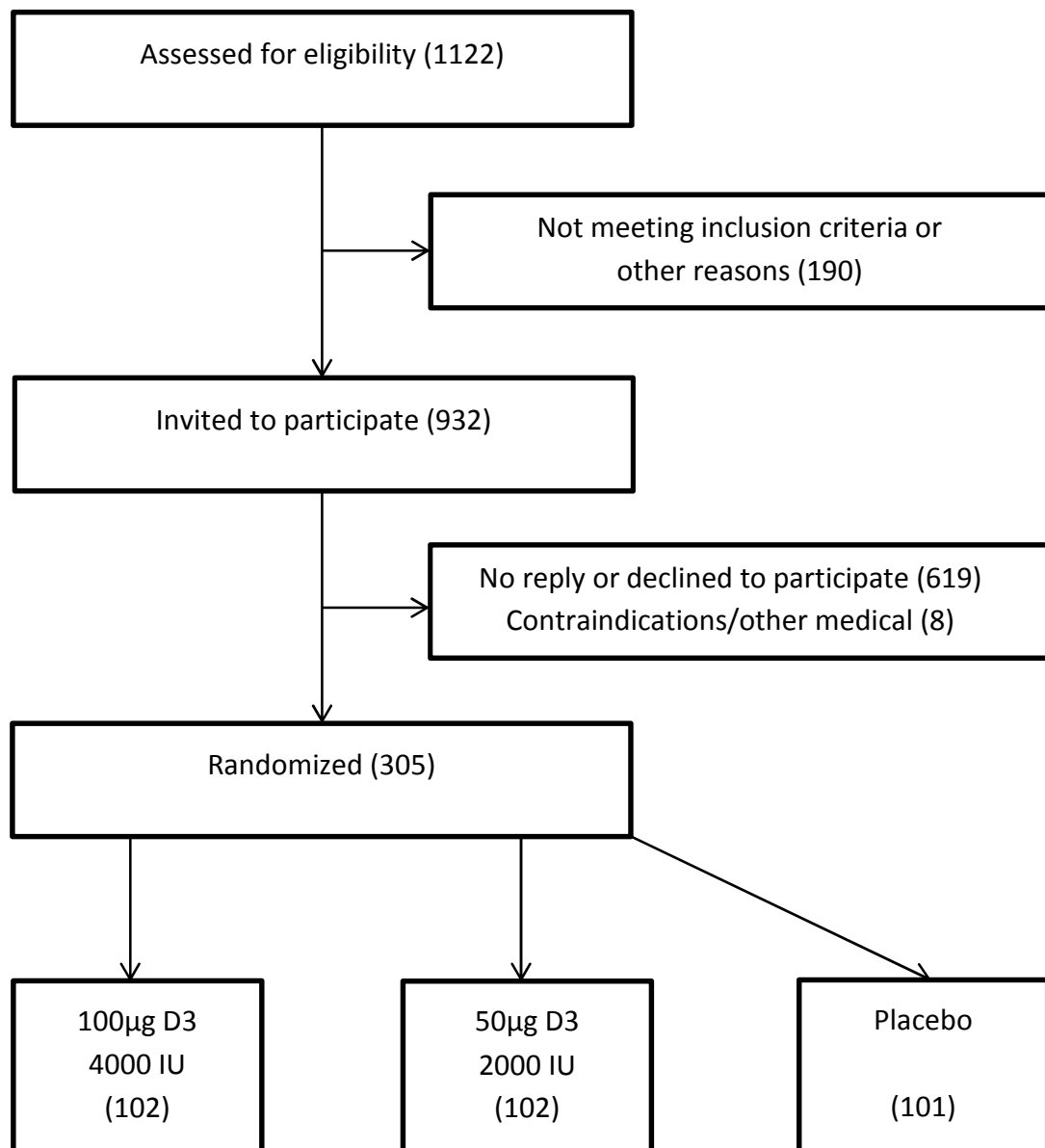
| Study Procedure | Month | | | |
|--|-------|---|---|----|
| | 0 | 1 | 6 | 12 |
| History | | | | |
| • Medical history and consent | x | | | |
| • Assessment of dietary calcium | x | | | |
| • Assessment of fractures and falls | x | | x | x |
| • Geriatric depression score (4 parts) | | | x | x |
| Physical measurements | | | | |
| • Height and weight | x | | | x |
| • Blood pressure, arterial stiffness | x | | x | x |
| • Grip strength, muscle pain | x | | x | x |
| • Short physical performance | | | | x |
| • Heel and wrist DEXA | | | | x |
| • Echocardiography | | | | x |
| Blood and urine collection | | | | |
| • Plasma biochemistry* | x | x | x | x |
| • RNA markers | x | | | x |
| • Buffy coats for DNA markers | x | | x | x |
| • Urinary albumin/creatinine | | | | x |
| Study treatment | | | | |
| • Issue study treatment | x | | x | |
| • Assess compliance | | | x | x |
| • Monitor adverse events | | | x | x |

* Total cholesterol, LDL-C, HDL-C, triglycerides, apolipoprotein A, apolipoprotein B. 25-hydroxy-vitaminD (25[OH]D); parathyroid hormone; (PTH), albumin, calcium, phosphate, alkaline phosphatase, creatinine, C-reactive protein (CRP); n-terminal brain natriuretic peptide (nBNP). Plasma biochemistry measurements at 1 month will be analyzed in a sub-set of 100 participants.

Table 2: Baseline characteristics of study participants, by allocated treatment

| Mean (SD) or % | 4000 IU/day (n=102) | 2000 IU/day (n=102) | Placebo (n=101) |
|------------------------------------|------------------------|------------------------|--------------------|
| Age, years | 71 (6) | 72 (6) | 72 (6) |
| Male | 51% | 50% | 51% |
| Current smoker | 7% | 7% | 7% |
| Dietary calcium (mg/day) | 724 (287) | 695 (292) | 713 (302) |
| Prior disease | | | |
| Heart disease* | 20% | 11% | 11% |
| Stroke/TIA | 5% | 8% | 6% |
| Hypertension | 39% | 43% | 35% |
| Diabetes | 9% | 9% | 9% |
| Fracture | 30% | 29% | 30% |
| Any fall in the past 6 months | 13% | 15% | 12% |
| Medication | | | |
| Any antihypertensive drugs | 43% | 49% | 40% |
| Statins | 29% | 27% | 21% |
| Any anti-platelet drugs | 18% | 19% | 15% |
| Vitamin D (<=400 IU/day) | 6% | 8% | 11% |
| Calcium | 2% | 1% | 3% |
| Physical measurements | | | |
| Height, cms | 168 (10) | 168 (10) | 167 (10) |
| Weight, kgs | 77 (17) | 78 (15) | 79 (15) |
| Body mass index, kg/m ² | 27 (5) | 27 (4) | 28 (5) |
| Grip strength, kg | 25 (11) | 25 (11) | 25 (11) |
| Systolic blood pressure, mm Hg | 132 (22) | 132 (17) | 129 (18) |
| Diastolic blood pressure, mm Hg | 77 (11) | 77 (10) | 76 (12) |

Figure 1: Selection of included participants in the BEST-D trial



Additional files provided with this submission:

Additional file 1: Web-Appendix (Supplementary Material)-F.docx, 21K

<http://www.trialsjournal.com/imedia/1080177683147369/supp1.docx>

Web-Appendix: Statistical Analysis Plan

1 Background

This Statistical Analysis Plan describes the outcomes, comparisons and statistical methods that will be used to assess the biochemical efficacy and safety of 100µg (4000 IU) and 50µg (2000 IU) vitamin D3 in the BEST-D trial. The nature of further secondary analyses and content of subsequent publications cannot be specified in detail but, where appropriate, the general analytical approach is described. All analyses and reports will be prepared by the Clinical Trial Service Unit, University of Oxford.

2 Outcomes

2.1 Co-primary outcomes

The two co-primary outcomes are:

- Mean plasma 25(OH)D concentrations at 12 months,
- Percentage of participants with a 25(OH)D concentration >90 nmol/L at 12 months.

2.2 Secondary outcomes

The secondary outcomes are:

- Mean plasma 25(OH)D concentrations at 1 and at 6 months;
- Percentage of participants with a 25(OH)D concentration >90 nmol/L at 1 and 6 months;
- Percentage of participants with a PTH concentration in the reference interval (1.1-6.8 pmol/L) at 1, 6 and 12 months;
- Percentage of participants with an albumin-corrected calcium concentration¹ above the reference interval (2.15-2.55 mmol/L) at 1, 6 and 12 months;
- Mean plasma concentrations at 6 and 12 months of albumin (g/L), phosphate (mmol/L), creatinine (mmol/L), and alkaline phosphatase (IU);
- Total cholesterol (mmol/L), LDL-C (mmol/L), HDL-C (mmol/L), triglycerides (mmol/L), apolipoprotein A (g/L) and apolipoprotein B (g/L) at 6 and 12 months;
- hsCRP at 6 and 12 months;
- NT-proBNP at 6 and 12 months;
- Systolic and diastolic blood pressure (mmHg), heart rate and arterial stiffness at 6 and 12 months;

¹ Calculated by calcium in mmol/L = 0.02 x (40 – plasma albumin [g/L])

- Echocardiographic measures of systolic function, diastolic function and global cardiac function (sub-set at 12 months only).

2.3 Tertiary outcomes

The tertiary outcomes are 12 month assessments of:

- Fractures at all sites and specific sites (n, %)
- Falls (n, %)
- Muscle pains (self-assessed visual analogue score 0-10)
- Physical activity (self-assessed visual analogue score 0-10)
- Joint pains (self-assessed visual analogue score 0-10)
- Respiratory infections (number)
- Geriatric Depression Score (1-4)
- Weight (kg)
- Height (cm)
- BMI (kg/m^2)
- Grip strength (kg)
- Short physical performance battery tests
 - 5 chair rises (sec)
 - Balance in tandem position (sec)
 - Balance in semi-tandem position (sec)
 - Balance side by side (sec)
 - 3 metre walk (sec)
- Bone density at the wrist (T-score and Z-score)
- Bone density at the heel (T-score and Z-score)
- Estimated GFR (ml/min/1.73 m^2)
- Urinary albumin/creatinine ratio.

3 Comparisons

3.1 Primary comparison of the co-primary outcomes

The primary comparison of the two co-primary outcomes (defined in section 2.1) will compare all participants allocated 100 µg vitamin D3 daily with all participants allocated 50 µg vitamin D3 daily.

3.2 Secondary comparisons of the co-primary outcomes

The secondary comparisons of the two co-primary outcomes will compare:

- Participants allocated 100 µg vitamin D3 daily versus participants allocated placebo;
- Participants allocated 50 µg vitamin D3 daily versus participants allocated placebo;
- Participants allocated any active vitamin D3 dose (50 µg or 100 µg daily) versus participants allocated placebo.

3.3 Tertiary comparisons of the co-primary outcomes

Tertiary comparisons of the two co-primary outcomes will compare participants allocated 100 µg vitamin D3 daily with participants allocated 50 µg vitamin D3 daily, separately by the following baseline characteristics:

- Sex (Male, Female)
- Age (<70, ≥70 years)
- BMI (<25, ≥25 to <30, ≥30 kg/m²)
- Plasma 25(OH)D concentrations (≤50, >50 nmol/L)
- Dietary calcium intake (≤700, >700 mg/day)
- eGFR calculated by the CKD-epi equation (≤75, >75 ml/min/1.73 m²)
- Prior history of cardiovascular disease² (Yes, No)

3.4 Comparisons of secondary and tertiary outcomes

All secondary outcomes will be compared between:

- Participants allocated 100 µg vitamin D3 daily versus participants allocated placebo
- Participants allocated 50 µg vitamin D3 daily versus participants allocated placebo
- Participants allocated any active vitamin D3 dose (50 µg or 100 µg daily) versus participants allocated placebo.

² Defined as a history of heart disease, stroke or TIA

Tertiary outcomes will only be formally compared between participants allocated any active vitamin D3 dose (50 µg or 100 µg daily) versus participants allocated placebo.

4 Safety and tolerability outcomes

The safety and tolerability of 100µg and 50µg vitamin D3 will be assessed from the following information.

4.1 Serious adverse events (SAEs)

All SAEs, regardless of whether the SAE is considered related to study treatment, will be recorded and grouped by body system or organ affected. The numbers and proportions of participants with SAEs in each group (vitamin D3 100µg, vitamin D3 50µg and placebo) will be described. SAEs of interest include:

- Incident cardiovascular disease events (myocardial infarction or stroke or any arterial revascularisation procedure);
- Incident cancer (any cancer excluding non-melanoma skin cancer);
- Death, hospitalisation, persistent or significant disability, or any important medical event, including hypercalcaemia or kidney stones.

Comparisons will include each individual dose versus placebo and both active doses combined versus placebo.

4.2 Reported reasons for stopping study treatment

All reasons for stopping treatment will be recorded and listed in relevant categories by treatment allocation. All adverse events, including non-serious adverse events, that cause participants to discontinue study treatment will be recorded and grouped by treatment allocation according to body system or organ. The numbers and proportions of participants in each treatment group with non-serious adverse events and serious adverse events that result in discontinuation of study treatment will be described.

4.3 Biochemical safety data

The biochemical safety data collected include calcium, phosphate, albumin, creatinine, alkaline phosphatase, and urinary albumin/creatinine. The analyses of these data are described in sections 2, 3 and 5.1.

5 Details of analyses

5.1 Methods of analysis

The assessments of efficacy and safety will involve comparisons among all randomized participants in their originally allocated treatment group irrespective of compliance during the scheduled treatment period (i.e., “intention-to-treat” analyses) (32, 33). Where a baseline value exists, between group comparisons of mean biomarker levels (or other physical measures) will be made by analysis of covariance, after adjustment for each participant’s baseline value (and, where necessary,

after transformation). Between group comparisons of proportions will be made using standard methods for 2x2 contingency tables. Missing values of particular biomarkers or physical measures will be imputed using multiple imputation (with 10 imputed data sets) with results across imputations being combined using the methods of Rubin. Results will be compared with those from complete-case analyses.

5.2 Allowance for multiplicity of comparisons

No allowance will be made for multiple comparisons in the primary assessment of the co-primary endpoints (section 3.1) and a two sided p value of <0.05 will be considered significant for each. For other analyses, allowance will be made in their interpretation for multiple hypothesis testing (32, 33) taking into account, if relevant, the type of measure and evidence from other studies. But, the more extreme the P-value (or, analogously, the further the lower limit of the confidence interval is from zero) after any allowance has been made for the nature of the particular comparison, the more reliable the comparison and, hence, the more definite any finding will be considered.

5.3 Tests for heterogeneity of effects

When a number of subgroups are considered, chance alone may lead to there being no apparent effect in several subgroups in which the effect of treatment really is about the same as that observed overall. In such circumstances, “lack of direct evidence of effect” is not good “evidence of lack of effect”, and clearly significant overall results would provide strong evidence of effects in some small subgroups where the results considered in isolation, are not conventionally significant. Instead, tests for heterogeneity (or, where appropriate, trend) in the effect of treatment allocation on differences in 25(OH)D between individuals allocated 100µg vitamin D3 and individuals allocated 50µg vitamin D3 will be performed to assess whether or not the effect in any particular subgroup is clearly different from the overall effect.

Web-Appendix References

A1: Collins R, MacMahon S. Reliable assessment of the effects of treatment on mortality and major morbidity, I: clinical trials. *Lancet*. 2001;357(9253):373-80.

NRES Committee South Central - Oxford B

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28 June 2012 (corrected: 06/08/2012)

Dr Jane Armitage
Clinical Trial Service Unit
University of Oxford
Richard Doll Building
Old Road Campus
Roosevelt Drive
Oxford
OX3 7LF

Dear Dr Armitage

| | |
|-------------------------|--|
| Study title: | BEST-D (Biochemical efficacy and safety trial of vitamin D): a dose-finding trial assessing biochemical and vascular effects of high dose vitamin D |
| REC reference: | 12/SC/0243 |
| Protocol number: | CTSU BEST-D |

Thank you for your letter of 11 May 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

| Document | Version | Date |
|---|-------------------------|---------------|
| Advertisement | 1.0, Poster | 04 April 2012 |
| Covering Letter | | 12 April 2012 |
| Evidence of insurance or indemnity | Sponsor Letter | 11 April 2012 |
| Evidence of insurance or indemnity | | 11 April 2012 |
| GP/Consultant Information Sheets | 1.0 | |
| Investigator CV | | 11 April 2012 |
| Investigator's Brochure | 1.0 | 23 March 2012 |
| Letter from Sponsor | Letter of Authorisation | 11 April 2012 |
| Letter of invitation to participant | 1.0 | 04 April 2012 |
| Other: Calcium Information | 1.0 | 11 April 2012 |
| Other: BEST-D Colour Logo | | 11 April 2012 |
| Other: CV for Dr Tomson | | 12 March 2012 |
| Other: Personal Safety Lone Worker Policy | 3 | 01 July 2008 |

| | | |
|---|-------------|---------------|
| Other: Short Geriatric Depression Scale | | |
| Participant Consent Form | 2.0 | 11 June 2012 |
| Participant Information Sheet | 2.0 | 11 June 2012 |
| Protocol | 1.1 | 11 June 2012 |
| REC application | 3.4 | 16 April 2012 |
| Response to Request for Further Information | | 11 May 2012 |
| Sample Diary/Patient Card | 1.0, D-STIL | 16 March 2012 |

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “*After ethical review – guidance for researchers*” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/SC/0243

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Prof Margaret Rees
Chair

Email: scsha.oxfordRECB@nhs.net

Enclosures: "After ethical review – guidance for researchers" [Emailed]

Copy to: Ms Heather House
heather.house@admin.ox.ac.uk

Mrs Dot Powers
dot.powers@seoxon-pct.nhs.uk

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From the Research and Commercial Services Office



THE QUEEN'S
ANNIVERSARY PRIZES
1994

Robert Clarke, MD, MRCP
Clinical Trial Service Unit
Harkness Building
Radcliffe Infirmary

Ref. RE/10/9/2

31 January 1997

Woodstock Road

Dear Dr Clarke,

Whitehall Study of London Civil Servants: 25 year follow-up survey

Thank you for writing to me in connection with indemnity for the above study.

I am grateful to you for the information which you provided, and have passed this directly to Mr Graham Waite, the University's insurance officer, who now deals with indemnity for clinical trials in conjunction with the University's insurers. (I suspect that he was not yet in post when you and I corresponded in connection with the pilot study.) I shall ask Mr Waite to get in touch with you as soon as possible.

Yours sincerely,

Catherine Quinn
Assistant Registrar

CWQ/CWQ